Method of Reducing Injury to Mammalian Cells

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FIELD OF THE INVENTION

This invention relates to methods of reducing the damaging effect of an injury to mammalian cells by treatment with compounds which reduce cell death or dysfunction, including cellular damage following episodes of tissue ischemia, trauma, epilepsy, and acute or chronic degeneration. The invention discloses methods of treating these disorders by administering inhibitors that disrupt protein-protein interactions involved in these disorders, screening methods to identify such inhibitors and specific compositions useful for treating these disorders.

BACKGROUND

Every year, stroke and neurotrauma afflict approximately 660,000 and 350,000 North American individuals, respectively, and about 175,000 stroke and 52,000 trauma victims will die (Stroke- American Stroke Association Web Site, 2000; Centers for Disease Control and Prevention, Traumatic injury in the United States: An interim report to Congress, Centers for Disease Control and Prevention, 2001). 4.5 million people live with Alzheimer's disease, with the prevalence expected to triple by 2050 (Hebert et al. (2003) Arch. Neurol. 60:1119-1122). Epilepsy is the third most common neurological disorder after stroke and Alzheimer's disease. It affects 2.3 million Americans of all ages. Approximately 181,000 new cases of seizures and epilepsy occur each year. One in every 10 Americans will experience a seizure at some point in their lives. Three percent will eventually develop epilepsy (Epilepsy Foundation of America Web Site, www.epilepsyfoundation.org/answerphone/statistics.cfm). Thus stroke, CNS trauma, neurodegenerative illnesses and epilepsy are each disorders of major public health significance. Moreover, cardiovascular disorders, pulmonary diseases, and endocrine illnesses such as diabetes count as among the most common causes of non-neurological morbidity and mortality worldwide. Common to all of these common illnesses is damage to cells in target tissues in the nervous, cardiovascular or endocrine system.

The public health consequences of these disorders are significant. For example, in 1998, $3.4 billion was paid in 1999 to just those Medicare beneficiaries that were discharged from short-stay hospitals, not including the long term care for >1,000,000 people that reportedly have functional limitations or difficulty with activities of daily living resulting from stroke (Heart and Stroke Statistics-2004 Update, American Heart Association, 2004). At this time, no therapeutics are available to reduce brain damage resulting from stroke, and this major disorder can be used as an example for the basis of the current invention, though the field of the invention obviously applies to other disorders involving mammalian cell injury.

Stroke is characterized by neuronal cell death in areas of ischemia, brain hemorrhage or trauma. Many lines of evidence have demonstrated that this cell death is triggered by glutamate over-excitation of neurons, leading to increased intracellular Ca^{2+} and increased nitric oxide due to an increase in nNOS (neuronal nitric oxide synthase) activity. Excitotoxicity is the process by which L-glutamate, the major excitatory neurotransmitter in the mammalian CNS, damages neurons (Olney (1969) Science 164:719-721; Olney and Sharpe (1969) Science 166:386-388). It is established as a predominant neurotoxic mechanism in acute neurological disorders such as stroke, epilepsy and traumatic nervous system injuries (reviewed in Rothman and Olney (1987) TINS 10:299-302; Choi et al. (1988) Neuron 8:623-634; Coyle and Puttfarcken (1993) Science 262:689-695; Lipton and Rosenberg (1994) N. Engl. J. Med. 330:613-622; Hardingham and Bading (2003) Trends. Neurosci. 26:81-89). In brain ischemia, excitotoxic activation of postsynaptic glutamate receptors triggers downstream pathways implicated in subsequent neuronal death (reviewed in Lipton (1999) Physiol. Rev. 79:1431-1568). Of these, Ca^{2+} influx through N-methyl- D-aspartate (NMDA) glutamate receptors was the process consistently revealed as a key event. In these studies, blocking NMDA receptors permitted neurons destined to die from anoxia to survive (Rothman (1983) Science 220:536-537, ; Goldberg et al. (1987) J. Pharmacol. Exp. Ther. 243:784-791), and animal research suggested that ischemic brain damage could be treated by this approach (Simon et al., (1984) Science 226:850-852). However, blocking NMDA receptors may be detrimental to animals and humans (Fix et al. (1993) Exp. Neurol. 123:204-215; Davis et al. (2000) Stroke 31:347-354; Ikonomidou et al. (2000) Proc. Natl. Acad. Sci. U.S.A. 97:12885-12890). Moreover, though blocking excitotoxicity was effective in laboratory models of disease, clinical trials of anti-excitotoxic therapies (AET) have generally failed to benefit patients (Davis et al., (1997) Lancet 349:32,

Davis et al. (2000) Stroke 31:347:354; Morris et al. (1999) J. Neurosurg 91:737-743; Lees et al. (2000) Lancet 355:1949-1954). The reason for this, in the face of a clear role for excitotoxicity in acute neurological disorders, has remained a mystery (Birmingham (2002) Nat. Med. 8:5; Ikonomidou and Turski (2002) Lancet Neurol. 1:383-386).

The present invention relates to our discovery that processes other than excitotoxicity are responsible for neuronal damage in conditions such as stroke. In neurons exposed to oxygen glucose deprivation (OGD), AET unmasks a lethal cation current I_{OGD} mediated by TRPM7, a member of the transient receptor potential (TRP) cation channel superfamily (Nadler et al. (2001) Nature 411:590-595). In OGD, I_{OGD} is activated by reactive oxygen/nitrogen species (ROS), permitting Ca^{2+} uptake that further stimulates ROS and I_{OGD} activation. Blocking I_{OGD} or suppressing TRPM7 expression prevents anoxic neuronal death *even in the absence of AET, indicating that TRPM7 is an essential mediator of anoxic death.* This work defines a new paradigm for understanding anoxic neuronal damage in which excitotoxicity is a subset of a larger framework of mammalian cell injury that involves TRP cation channels.

SUMMARY

The present invention relates to the treatment of cytodestructive disorders such as tissue ischemia or brain damage resulting from stroke, epilepsy, neurodegenerative conditions or traumatic brain and spinal cord injuries by modulating specific protein:protein interactions including, but not limited to, PDZ domain:PDZ Ligand (PL) interactions that involve, directly or indirectly, members of the TRP cation channel family, and are involved in mediating these clinical disorders. Methods for identifying specific therapeutics that modulate the specific protein:protein interactions involved in these disorders are also provided. Compounds and compositions for treating these neuronal disorders are also disclosed.

Methods of identifying the cellular PDZ proteins that are bound by TRP channels and associated proteins are provided herein after speaking with Dr. M. Tymianski (MT) it was determined that although we would remove specific reference to PLC, we would keep general statements regarding TRP channels and associated proteins. Methods are also provided to identify inhibitors that are high affinity for TRP-specific interactions as well as TRP-associated protein interactions. Other methods are provided to determine selectivity of inhibition, both

against the different TRP channels, TRP-associated proteins and the PDZs that can bind them. Methods for delivering peptide inhibitors to cells such as neuron cells are also disclosed.

One class of pharmaceutical compositions that are provided include a pharmaceutical composition comprising an isolated, recombinant or synthetic polypeptide inhibitor that inhibits binding between a TRP channel and a PDZ protein with a physiologically acceptable carrier, diluent or excipient, wherein the polypeptide comprises a C-terminal amino acid sequence of X-L/V/I-X-V/L/A. In certain embodiments, the C-terminal amino acid sequence of the polypeptide is XLML (SEQ ID NO:1). These compositions can be used to inhibit binding between a TRP channel and various PDZ proteins, including, for example, but not limited to, NNOS, LIM, KIAA1095, HEMBA1003117, AIPC, KIAA1526, DVL1, DVL2, DVL3, PTPL1, ZO-1, ZO-2, ZO-3, KIAA1719, Mupp1, INADL, Shank 3, MINT1, MINT2, MAGI1, MAGI2, MAGI3, NeDLG, syntenin, PSD-95, hDLG, PAR3, MAST1, MAST2, AF6, SIP1, LIM mystique, HTRA2, TIP-1, KIAA0316, PICK1, RIM-2, INADL, Syntrophin 1 alpha , SITAC-18, PAR3L, MAST2, and NSP [novel serine protease].

The polypeptides in these compositions can be of varying lengths. In a certain embodiment such polypeptides are 3-20 amino acids in length. In other embodiments, the polypeptides are fusion polypeptides, which include the C-terminal amino acid sequence of the PL polypeptide and a segment of a transmembrane transporter sequence that is effective to facilitate transport of the polypeptide into the desired cell type, for example a neuronal cell (also known as a cell-membrane transduction domain).

Another class of pharmaceutical compositions may also include an isolated, recombinant or synthetic polypeptide and a physiologically acceptable carrier, diluent or excipient, wherein the polypeptide is 3-20 amino acids in length and inhibits binding between a TRP channel and a PDZ protein. The polypeptides in some of these compositions are 3-8 amino acids in length. Exemplary sequences of such polypeptides include LML

Still other pharmaceutical compositions include a fusion polypeptide that inhibits binding between a TRP channel and a PDZ protein and a physiologically acceptable carrier, diluent or excipient. In certain embodiments, the fusion polypeptide inhibitor in these compositions is a fusion of (i) a 9 amino acid segment that has a C-terminal sequence and (ii) an

amino acid segment of a transmembrane transporter that is effective to transport the polypeptide into a neuron or other affected cell. In other embodiments the fusion polypeptide inhibitor comprises (i) a 3-8 amino acid segment that has a C-terminal sequence and (ii) an amino acid segment of a transmembrane transporter that is effective to transport the polypeptide into a neuron or other affected cell. In yet other embodiments the fusion polypeptide inhibitor comprises (i) a 9-20 amino acid segment that has a C-terminal sequence and (ii) an amino acid segment of a transmembrane transporter that is effective to transport the polypeptide into a neuron or other affected cell.

The polypeptide inhibitors in the foregoing pharmaceutical compositions can be used in a variety of therapies, including treatment of a number of neurological disorders. Examples of such disorders include, but are not limited to, stroke, ischemia, myocardial ischemia, glaucoma, Parkinson's disease, Huntington's disease, Alzheimer's disease, epilepsy and inherited ataxias. The inhibitors can also be used in the preparation of medicaments for use in the treatment of neurological disorders.

Still other pharmaceutical compositions include small inhibitory RNA ("siRNA") sequences that can specifically reduce the activity of a TRP cation channel family member and protect against cell death from OGD. Specific inhibitory sequences and methods of identifying siRNA sequences are described within.

Also provided are methods for determining whether a test compound modulates binding between a PDZ protein and a TRP channel. Certain of these methods involve contacting a PDZ–domain containing polypeptide and a PDZ-Ligand ("PL") containing peptide having at least the C-terminal 3 amino acids of the TRP channel in the presence of the test compound. In certain embodiments, the PDZ proteins in these screening methods may be selected from the group consisting of DLG1, DLG2, KIAA0973, NeDLG, Outermembrane protein, Syntrophin alpha 1, TIP1, TIP2, INADL, KIAA0807, KIAA1634, Lim-Mystique, LIM-RIL, MAGI1, MAGI2, NH-ERF1, NH-ERF2, Syntrophin beta-1 and Syntrophin gamma-1,RIM-2 , Mint 1, Syntrophin 1 alpha , SITAC-18, ZO-1, PAR3L, MAST2, PAR3, and NSP [novel serine protease]The amount (Susan do you think that amount a broader term than concentration? OK) of complex formed between the PDZ-domain polypeptide and the PL peptide is then determined.

The test compound is identified as a potential inhibitor of binding between the PDZ protein and the TRP channel if a lower amount of the complex is detected in the presence of the test compound relative to the concentration of the complex in the absence of the test compound. Another assay can be conducted using compounds identified in the initial screen to determine whether the identified compound mitigates against a condition associated with a neuronal or ischemic disorder. Examples of such assays include necrosis assays, apoptosis assays, caspase assays, cytochrome c assays and cell lysis assays.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 shows a phylogenetic tree of TRP channels

FIGURE 2. shows the domain structures of different TRP channel sub-families

FIGURE 3 demonstrates that siRNA knockdown of TRPM7 reduces Reactive Oxygen Species (ROS) production, calcium uptake and cell death under prolonged oxygen-glucose deprivation (OGD).

FIGURE 4 shows the loss of neuroprotection and blockage of Ca^{2+} accumulation by glutamate and Ca^{2+} channel inhibitors with extended OGD.

FIGURE 5 shows the delay of calcium uptake in the presence of glutamate and Ca^{2+} channel inhibitors and the effect of extracellular calcium concentration on OGD survival.

FIGURE 6 shows the effect of Gd^{3+} on calcium uptake in the presence of glutamate and Ca^{2+} channel inhibitors

FIGURE 7 demonstrates that Gd^{3+} can permit survival of neurons destined to die from prolonged OGD, and that superoxide and nNOS inhibition are synergistic while inhibitors of ROS pathways outside of superoxide and nitric oxide show no benefit in OGD survival.

FIGURE 8 shows the results of rtPCR experiments demonstrating the effect of siRNA treatment of TRPM7 on other TRPM channels using different constructs.

FIGURES 9A, 9B, 9C, AND 9D show the results of titrations of the Tat-TRPM7 peptide with RIM-2 d2, INADL d3, ZO-1 d2, and Par3 d3.

FIGURES 10A AND 10B show the results of titrations of Peptide #1829 with ZO-1 d2 and Peptide #1839 with INADL d3.

FIGURE 11 shows the adenoviral construct. The TRPM7 siRNA-pAdTrack[(1)] (containing the hairpin under the H1 promoter and GFP under a CMV promoter as well as adeno recombination sequences was cotransfected with pAdEasy (containing viral sequences) into HEK cells and the cells selected with kanamycin. Recombined virus containing the TRPM7 siRNA hairpin and GFP sequences was then be produced from these cells.

(Reference (1): T. C. He, S. Zhou, L. T. da Costa, J. Yu, K. W. Kinzler, and B. Vogelstein. A simplified system for generating recombinant adenoviruses. *Proc.Natl.Acad.Sci.U.S.A* 95 (5):2509-2514, 1998).

FIGURE 12 shows the effects of infecting primary cultured neurons on TRPM7 mRNA by RT-PCR. Cultured cortical neurons plated in 12 well plates (106 cells/well) were infected at the time of plating with the corresponding adenoviral constructs and the RNA was harvested 5 days later. RT-PCR was performed on equivalent amounts of RNA using primers for TRPM7 or β-actin.

FIGURE 13 shows the effect of oxygen glucose deprivation (OGD) for the indicated duration on neuronal cell death in the presence (MCN(+)) or absence (MCN(-)) of the combination of MK-801 (10 μM), CNQX (10 μM) and nimodipine (2 μM), denoted as MCN, antagonists of NMDA and AMPA/kainate glutamate receptors and L-type Ca^{2+} channels, respectively. The fraction of dead cells was determining by dividing the number of neurons expressing GFP which became stained with propidium iodide 20h after OGD by the number of neurons expressing GFP at the beginning of the experiment. Data for each culture was obtained from counting cells in 3 high power microscope fields per experiment.

FIGURE 14 shows the effect of treating the cultures with Tat-9cTRPM7. The sequence of Tat-9cTRPM7 is: [Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Ser-Thr-Asn-Ser-Val-Arg-Leu-Met-Leu] or [YGRKKRRQRRR-STNSVRLML], whereby the first 11 residues correspond to the cell membrane transduction domain of the human immunodeficiency virus type 1 (HIV-1) Tat protein and the last 9 residues correspond to the last 9 amino acids of the C-terminus of human TRPM7 (accession Q96QT4). We predict similar results with a Tat-conjugated peptide encoding the last 9 residues of the mouse TRPM7 C-terminus (YGRKKRRQRRR-ATNSVRLML; accession Q923J1). (A) Neuronal survival at 20h in the indicated concentrations of Tat-9cTRPM7 in the absence of excitotoxic challenge.(B) Neuronal survival 20h after challenging the cultures for 1h with the indicated concentration of NMDA. Tat-9cTRPM7 was applied immediately after the NMDA challenge.

BRIEF DESCRIPTION OF THE TABLES

TABLE 1 shows classifications of genetically encoded and non-encoded amino acids. Column 1shows the classification, column 2 shows genetically encoded amino acids, and column 3 genetically non-encoded amino acids.

TABLE 2 shows glutamate receptors with PL sequences. Column 1 shows the names of the receptor, column 2 shows the GenBank number, column 3 shows the C-terminal 20mer sequence, column 4 shows the C-terminal 4mer sequence, column 5 shows which sequences are PLs, and column 6 shows internal PL ID.

TABLE 3 shows the number of TRP genes in C. elegans, Drosophila melangaster, mice, and humans. Column 1 shows the subfamily, column 2 shows the number in C. elegans, column 3 shows the number in Drosophila melanogaster, column 4 shows the number in mice, and column 5 shows the number in humans.

TABLE 4 shows the nomenclature of the mammalian TRP superfamily. Column 1 shows the name of the protein, column 2shows the group number, column 3 shows former names of the protein, and column 4 shows the GenBank accession numbers.

TABLE 5 shows the sequences of PDZ domains cloned to produce GST-PDZ fusion proteins. Column 1 shows the gene name, column 2 shows the GenBank GI or Accession number, column 3 shows the domain number, and column 4 shows the sequence fused to the GST construct.

TABLE 6 shows PDZs predicted to interact with TRPM7

TABLE 7 shows PDZs demonstrated to interact with specific PL proteins.

TABLE 8 shows PDZ domains which bind the Tat-TRPM7 peptide in G0 or G3 assays. Column 1 shows the name of the PDZ protein, column 2 shows the domain number, column 3 shows the GenBank number for the gene encoding the PDZ protein, column 4 shows whether the literature indicates expression in the nervous system or brain, and column 5 shows the literature reference for column 5.

TABLE 9 shows the results of titrations of the Tat-TRPM7 peptide with RIM2 (177.4), Mint 1 (d1,d2) (36.5a), TIP1 d1 (54.10), Mint1 d1 (146.5), Mint1 d2 (147.2), INADL d3 (96.3), MUPP1 d3 (108.3), Syntrophin 1 alpha d1 (52.5), SITAC-18 d1 (122.2), SITAC-18 d2(123.2), LIM Mystique d1 (232.1), ZO-1 d2 (241.3), PAR3L d3 (406.1), MAST2 d1 (174.6),

PAR3 d3 (278.1), and KIAA1284 d1 (191.2), Column 1 shows the PDZ protein and domain number, column 2 shows the EC50 in μM, column 3 shows the error of fit for the EC_{in} in μM, and column 4 shows the OD_{max} 450 nm). ND=not determined.

TABLE 10 shows the results of titration of peptides with Z0-1 d2 and INADL d3. Column 1 shows peptide #, column 2 shows peptide sequence, column 3 shows SEQ ID NO of the peptide, column 4 shows the PDZ protein and domain number, column 5 shows the EC50 in μM, column 6 shows the error of fit for the EC_{in} in μM, and column 7shows the OD_{max} 450 nm). ND=not determined.

TABLE 11 shows the results of titrations or binding assays of peptides with RIM-2 d1, INADL d3, ZO-1 d2, PAR3 d3, and syntrophin1 alpha d1. Column 1 shows PDZ domain bound, Column 2 shows peptide sequence, and Column 3 shows SEQ ID NO.

DETAILED DESCRIPTION

I. Definitions

"Polypeptide," "protein" and "peptide" are used interchangeably herein and include a molecular chain of amino acids linked through peptide bonds. The terms do not refer to a specific length of the product. Thus, "peptides," "oligopeptides," and "proteins" are included within the definition of polypeptide. The terms include post-translational modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like. In addition, protein fragments, analogs, mutated or variant proteins, fusion proteins and the like are included within the meaning of polypeptide. The polypeptide, protein and peptides may be in cyclic form or they may be in linear form (Piserchio et al. Chem. Biol. (2004) 111:469-473; Li et al., Bioorg. Med. Chem. Lett. (2004) 14:13855-1388; Baruch et al. Biochemistry (2003) 42:2797-2805; Harris et al., Biochemistry (2001) 40:5921-5930).

A "fusion protein" or "fusion polypeptide" as used herein refers to a composite protein, i.e., a single contiguous amino acid sequence, made up of two (or more) distinct, heterologous polypeptides which are not normally fused together in a single amino acid sequence. Thus, a fusion protein can include a single amino acid sequence that contains two entirely distinct amino acid sequences or two similar or identical polypeptide sequences, provided that these sequences are not normally found together in the same configuration in a

single amino acid sequence found in nature. Fusion proteins can generally be prepared using either recombinant nucleic acid methods, i.e., as a result of transcription and translation of a recombinant gene fusion product, which fusion comprises a segment encoding a polypeptide of the invention and a segment encoding a heterologous protein, or by chemical synthesis methods well known in the art.

A "fusion protein construct" as used herein is a polynucleotide encoding a fusion protein.

As used herein, the term "PDZ domain" refers to protein sequence (i.e., modular protein domain) of approximately 90 amino acids, characterized by homology to the brain synaptic protein PSD-95, the Drosophila septate junction protein Discs-Large (DLG), and the epithelial tight junction protein ZO1 (ZO1). PDZ domains are also known as Discs-Large homology repeats ("DHRs") and GLGF repeats. PDZ domains generally appear to maintain a core consensus sequence (Doyle, D. A., 1996, *Cell* 85: 1067-76).

PDZ domains are found in diverse membrane-associated proteins including members of the MAGUK family of guanylate kinase homologs, several protein phosphatases and kinases, neuronal nitric oxide synthase, and several dystrophin-associated proteins, collectively known as syntrophins.

Exemplary PDZ domain-containing proteins and PDZ domain sequences are shown in **TABLE 5**. The term "PDZ domain" also encompasses variants (e.g., naturally occurring variants) of the sequences of **TABLE 5** (e.g., polymorphic variants, variants with conservative substitutions, and the like). Typically, PDZ domains are substantially identical to those shown in **TABLE 5**, e.g., at least about 70%, at least about 80%, or at least about 90% amino acid residue identity when compared and aligned for maximum correspondence.

As used herein, the term "PDZ protein" refers to a naturally occurring protein containing a PDZ domain. Exemplary PDZ proteins include, but are not limited to NNOS, LIM, KIAA1095, HEMBA1003117, AIPC, KIAA1526, DVL1, DVL2, DVL3, PTPL1, ZO-1, ZO-2, ZO-3, KIAA1719, Mupp1, INADL, Shank 3, MINT1, MINT2, MAGI1, MAGI2, MAGI3, NeDLG, syntenin, PSD-95, hDLG, PAR3, MAST1, MAST2, AF6, SIP1, LIM mystique, ,HTRA2, TIP-1, KIAA0316, PICK1, RIM-2, INADL, Syntrophin 1 alpha , SITAC-18, PAR3L, MAST2, and NSP [novel serine protease]and those listed in **TABLE** 5.

As used herein, the term "PDZ-domain polypeptide" refers to a polypeptide containing a PDZ domain, such as a fusion protein including a PDZ domain sequence, a naturally occurring PDZ protein, or an isolated PDZ domain peptide.

As used herein, the term "PL protein" or "PDZ Ligand protein" refers to a naturally occurring protein that forms a molecular complex with a PDZ-domain, or to a protein whose carboxy-terminus, when expressed separately from the full length protein (e.g., as a peptide fragment of 4-25 residues, e.g., 8, 10, 12, 14 or 16 residues), forms such a molecular complex. The molecular complex can be observed *in vitro* using the A assay or G assay described *infra*, or *in vivo*. Exemplary TRP channel PL proteins listed in **TABLE 4** and TRP associated proteins listed in **TABLE 2** are demonstrated to bind specific PDZ proteins. This definition is not intended to include anti-PDZ antibodies and the like.

As used herein, the terms "NMDA receptor," "NMDAR," or "NMDA receptor protein" refer to a membrane associated protein that is known to interact with NMDA. The term thus includes the various subunit forms, including for example, those listed in **TABLE 2.** The receptor can be a non-human mammalian NMDAR (e.g., mouse, rat, rabbit, monkey) or a human NMDAR, for example.

As used herein, the term "NMDAR-PL" or "NMDA receptor-PL" refers to a NMDA receptor that forms a molecular complex with a PDZ domain or to a NMDAR protein whose carboxy-terminus, when expressed separately from the full length protein (e.g., as a peptide fragment of 4-25 residues, e.g., 8, 10, 12, 14 or 16 residues), forms such a molecular complex.

As used herein, the term "TRP channel" refers to an ion channel protein of the transient receptor potential family of proteins.

As used herein, the term "TRP associated proteins" refers to proteins that interact physically or functionally with TRP [ion] channel proteins. MT states that he will soon have data that demonstrates binding between TRPM7 and NMDAR (a TRP associated protein).

As used herein, the term "ion channel" refers to an ion channel protein, which could refer singularly or collectively to different ion channels, including TRP channels, NMDA Receptor channels, or other ion channels.

As used herein, "oxygen-glucose deprivation" or "OGD" refers to a change in the cellular environment that results in lower than normal access to oxygen and/or glucose.

As used herein, a "PL sequence" refers to the amino acid sequence of the C-terminus of a PL protein (e.g., the C-terminal 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 20 or 25 residues) ("C-terminal PL sequence") or to an internal sequence known to bind a PDZ domain ("internal PL sequence").

As used herein, a "PL peptide" is a peptide having a sequence from, or based on, the sequence of the C-terminus of a PL protein. Exemplary PL peptides (biotinylated) are listed in **TABLE 2** and **TABLE 4**.

As used herein, a "PL fusion protein" is a fusion protein that has a PL sequence as one domain, typically as the C-terminal domain of the fusion protein. An exemplary PL fusion protein is a tat-PL sequence fusion.

As used herein, the term "PL inhibitor peptide sequence" refers to PL peptide amino acid sequence that (in the form of a peptide or PL fusion protein) inhibits the interaction between a PDZ domain polypeptide and a PL peptide (e.g., in an A assay or a G assay).

As used herein, a "PDZ-domain encoding sequence" means a segment of a polynucleotide encoding a PDZ domain. In various embodiments, the polynucleotide is DNA, RNA, single stranded or double stranded.

As used herein, the terms "antagonist" and "inhibitor," when used in the context of modulating a binding interaction (such as the binding of a PDZ domain sequence to a PL sequence), are used interchangeably and refer to an agent that reduces the binding of the, e.g., PL sequence (e.g., PL peptide) and the, e.g., PDZ domain sequence (e.g., PDZ protein, PDZ domain peptide).

As used herein, the terms "agonist" and "enhancer," when used in the context of modulating a binding interaction (such as the binding of a PDZ domain sequence to a PL sequence), are used interchangeably and refer to an agent that increases the binding of the, e.g., PL sequence (e.g., PL peptide) and the, e.g., PDZ domain sequence (e.g., PDZ protein, PDZ domain peptide).

The terms "isolated" or "purified" means that the object species (e.g., a polypeptide) has been purified from contaminants that are present in a sample, such as a sample obtained from natural sources that contain the object species. If an object species is isolated or purified it is the predominant macromolecular (e.g., polypeptide) species present in a sample (i.e., on a molar basis it is more abundant than any other individual species in the composition),

and preferably the object species comprises at least about 50 percent (on a molar basis) of all macromolecular species present. Generally, an isolated, purified or substantially pure composition comprises more than 80 to 90 percent of all macromolecular species present in a composition. Most preferably, the object species is purified to essential homogeneity (i.e., contaminant species cannot be detected in the composition by conventional detection methods), wherein the composition consists essentially of a single macromolecular species.

The term "recombinant" when used with respect to a polypeptide refers to a polypeptide that has been prepared be expressing a recombinant nucleic acid molecule in which different nucleic acid segments have been joined together using molecular biology techniques.

The term "synthesized" when used with respect to a polypeptide generally means that the polypeptide has been prepared by means other than simply purifying the polypeptide from naturally occurring sources. A synthesized polypeptide can thus be prepared by chemical synthesis, recombinant means, or by a combination of chemical synthesis and recombinant means. Segments of a synthesized polypeptide, however, may be obtained from naturally occurring sources.

The term "biological function" or "biological activity" in the context of a cell, refers to a detectable biological activity normally carried out by the cell, e.g., a phenotypic change such as proliferation, cell activation, excitotoxicity responses, neurotransmitter release, cytokine release, degranulation, tyrosine phosphorylation, ion (e.g., calcium) flux, metabolic activity, apoptosis, changes in gene expression, maintenance of cell structure, cell migration, adherence to a substrate, signal transduction, cell-cell interactions, and others described herein or known in the art.

As used herein, the terms "peptide mimetic," "peptidomimetic," and "peptide analog" are used interchangeably and refer to a synthetic chemical compound which has substantially the same structural and/or functional characteristics of a PL inhibitory or PL binding peptide of the invention. The mimetic can be either entirely composed of synthetic, non-natural analogues of amino acids, or is a chimeric molecule of partly natural peptide amino acids and partly non-natural analogs of amino acids. The mimetic can also incorporate any amount of natural amino acid conservative substitutions as long as such substitutions also do not substantially alter the mimetic's structure and/or inhibitory or binding activity. As with polypeptides of the invention which are conservative variants, routine experimentation will

determine whether a mimetic is within the scope of the invention, i.e., that its structure and/or function is not substantially altered. Thus, a mimetic composition is within the scope of the invention if it is capable of binding to a PDZ domain and/or inhibiting a PL-PDZ interaction.

Polypeptide mimetic compositions can contain any combination of nonnatural structural components, which are typically from three structural groups: a) residue linkage groups other than the natural amide bond ("peptide bond") linkages; b) non-natural residues in place of naturally occurring amino acid residues; or c) residues which induce secondary structural mimicry, i.e., to induce or stabilize a secondary structure, e.g., a beta turn, gamma turn, beta sheet, alpha helix conformation, and the like.

A polypeptide can be characterized as a mimetic when all or some of its residues are joined by chemical means other than natural peptide bonds. Individual peptidomimetic residues can be joined by peptide bonds, other chemical bonds or coupling means, such as, e.g., glutaraldehyde, N-hydroxysuccinimide esters, bifunctional maleimides, N,N=-dicyclohexylcarbodiimide (DCC) or N,N=-diisopropylcarbodiimide (DIC). Linking groups that can be an alternative to the traditional amide bond ("peptide bond") linkages include, e.g., ketomethylene (e.g., -C(=O)-CH_2- for -C(=O)-NH-), aminomethylene (CH_2-NH), ethylene, olefin (CH=CH), ether (CH_2-O), thioether (CH_2-S), tetrazole (CN_4-), thiazole, retroamide, thioamide, or ester (see, e.g., Spatola (1983) in *Chemistry and Biochemistry of Amino Acids, Peptides and Proteins*, Vol. 7, pp 267-357, *A Peptide Backbone Modifications*, Marcel Dekker, NY).

A polypeptide can also be characterized as a mimetic by containing all or some non-natural residues in place of naturally occurring amino acid residues. Nonnatural residues are well described in the scientific and patent literature; a few exemplary nonnatural compositions useful as mimetics of natural amino acid residues and guidelines are described below.

Mimetics of aromatic amino acids can be generated by replacing by, e.g., D- or L-naphylalanine; D- or L- phenylglycine; D- or L-2 thieneylalanine; D- or L-1, -2, 3-, or 4-pyreneylalanine; D- or L-3 thieneylalanine; D- or L-(2-pyridinyl)-alanine; D- or L-(3-pyridinyl)-alanine; D- or L-(2-pyrazinyl)-alanine; D- or L-(4-isopropyl)-phenylglycine; D-(trifluoromethyl)-phenylglycine; D-(trifluoromethyl)-phenylalanine; D-p-fluorophenylalanine; D- or L-p-biphenylphenylalanine; K- or L-p-methoxybiphenylphenylalanine; D- or L-2-indole(alkyl)alanines; and, D- or L-alkylainines, where alkyl can be substituted or unsubstituted

methyl, ethyl, propyl, hexyl, butyl, pentyl, isopropyl, iso-butyl, sec-isotyl, iso-pentyl, or a non-acidic amino acids. Aromatic rings of a nonnatural amino acid include, e.g., thiazolyl, thiophenyl, pyrazolyl, benzimidazolyl, naphthyl, furanyl, pyrrolyl, and pyridyl aromatic rings.

Mimetics of acidic amino acids can be generated by substitution by, e.g., non-carboxylate amino acids while maintaining a negative charge; (phosphono)alanine; sulfated threonine. Carboxyl side groups (e.g., aspartyl or glutamyl) can also be selectively modified by reaction with carbodiimides (R=-N-C-N-R=) such as, e.g., 1-cyclohexyl-3(2-morpholinyl-(4-ethyl) carbodiimide or 1-ethyl-3(4-azonia- 4,4- dimetholpentyl) carbodiimide. Aspartyl or glutamyl can also be converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Mimetics of basic amino acids can be generated by substitution with, e.g., (in addition to lysine and arginine) the amino acids ornithine, citrulline, or (guanidino)-acetic acid, or (guanidino)alkyl-acetic acid, where alkyl is defined above. Nitrile derivative (e.g., containing the CN-moiety in place of COOH) can be substituted for asparagine or glutamine. Asparaginyl and glutaminyl residues can be deaminated to the corresponding aspartyl or glutamyl residues.

Arginine residue mimetics can be generated by reacting arginyl with, e.g., one or more conventional reagents, including, e.g., phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, or ninhydrin, preferably under alkaline conditions.

Tyrosine residue mimetics can be generated by reacting tyrosyl with, e.g., aromatic diazonium compounds or tetranitromethane. N-acetylimidizol and tetranitromethane can be used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively.

Cysteine residue mimetics can be generated by reacting cysteinyl residues with, e.g., alpha-haloacetates such as 2-chloroacetic acid or chloroacetamide and corresponding amines; to give carboxymethyl or carboxyamidomethyl derivatives. Cysteine residue mimetics can also be generated by reacting cysteinyl residues with, e.g., bromo-trifluoroacetone, alpha-bromo-beta-(5-imidozoyl) propionic acid; chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide; methyl 2-pyridyl disulfide; p-chloromercuribenzoate; 2-chloromercuri-4 nitrophenol; or, chloro-7-nitrobenzo-oxa-1,3-diazole.

Lysine mimetics can be generated (and amino terminal residues can be altered) by reacting lysinyl with, e.g., succinic or other carboxylic acid anhydrides. Lysine and other alpha-amino-containing residue mimetics can also be generated by reaction with imidoesters, such as

methyl picolinimidate, pyridoxal phosphate, pyridoxal, chloroborohydride, trinitrobenzenesulfonic acid, O-methylisourea, 2,4, pentanedione, and transamidase-catalyzed reactions with glyoxylate.

Mimetics of methionine can be generated by reaction with, e.g., methionine sulfoxide. Mimetics of proline include, e.g., pipecolic acid, thiazolidine carboxylic acid, 3- or 4-hydroxy proline, dehydroproline, 3- or 4-methylproline, or 3,3,-dimethylproline. Histidine residue mimetics can be generated by reacting histidyl with, e.g., diethylprocarbonate or para-bromophenacyl bromide.

Other mimetics include, e.g., those generated by hydroxylation of proline and lysine; phosphorylation of the hydroxyl groups of seryl or threonyl residues; methylation of the alpha-amino groups of lysine, arginine and histidine; acetylation of the N-terminal amine; methylation of main chain amide residues or substitution with N-methyl amino acids; or amidation of C-terminal carboxyl groups.

A component of a natural polypeptide (e.g., a PL polypeptide or PDZ polypeptide) can also be replaced by an amino acid (or peptidomimetic residue) of the opposite chirality. Thus, any amino acid naturally occurring in the L-configuration (which can also be referred to as the R or S, depending upon the structure of the chemical entity) can be replaced with the amino acid of the same chemical structural type or a peptidomimetic, but of the opposite chirality, generally referred to as the D- amino acid, but which can additionally be referred to as the R- or S- form.

The mimetics of the invention can also include compositions that contain a structural mimetic residue, particularly a residue that induces or mimics secondary structures, such as a beta turn, beta sheet, alpha helix structures, gamma turns, and the like. For example, substitution of natural amino acid residues with D-amino acids; N-alpha-methyl amino acids; C-alpha-methyl amino acids; or dehydroamino acids within a peptide can induce or stabilize beta turns, gamma turns, beta sheets or alpha helix conformations. Beta turn mimetic structures have been described, e.g., by Nagai (1985) Tet. Lett. 26:647-650; Feigl (1986) J. Amer. Chem. Soc. 108:181-182; Kahn (1988) J. Amer. Chem. Soc. 110:1638-1639; Kemp (1988) Tet. Lett. 29:5057-5060; Kahn (1988) J. Molec. Recognition 1:75-79. Beta sheet mimetic structures have been described, e.g., by Smith (1992) J. Amer. Chem. Soc. 114:10672-10674. For example, a type VI beta turn induced by a cis amide surrogate, 1,5-disubstituted tetrazol, is described by

Beusen (1995) Biopolymers 36:181-200. Incorporation of achiral omega-amino acid residues to generate polymethylene units as a substitution for amide bonds is described by Banerjee (1996) Biopolymers 39:769-777. Secondary structures of polypeptides can be analyzed by, e.g., high-field 1H NMR or 2D NMR spectroscopy, see, e.g., Higgins (1997) J. Pept. Res. 50:421-435. See also, Hruby (1997) Biopolymers 43:219-266, Balaji, et al., U.S. Patent No. 5,612,895.

As used herein, "peptide variants" and "conservative amino acid substitutions" refer to peptides that differ from a reference peptide (e.g., a peptide having the sequence of the carboxy-terminus of a specified PL protein) by substitution of an amino acid residue having similar properties (based on size, polarity, hydrophobicity, and the like). Thus, insofar as the compounds that are encompassed within the scope of the invention are partially defined in terms of amino acid residues of designated classes, the amino acids may be generally categorized into three main classes: hydrophilic amino acids, hydrophobic amino acids and cysteine-like amino acids, depending primarily on the characteristics of the amino acid side chain. These main classes may be further divided into subclasses. Hydrophilic amino acids include amino acids having acidic, basic or polar side chains and hydrophobic amino acids include amino acids having aromatic or apolar side chains. Apolar amino acids may be further subdivided to include, among others, aliphatic amino acids. The definitions of the classes of amino acids as used herein are as follows:

"Hydrophobic Amino Acid" refers to an amino acid having a side chain that is uncharged at physiological pH and that is repelled by aqueous solution. Examples of genetically encoded hydrophobic amino acids include Ile, Leu and Val. Examples of non-genetically encoded hydrophobic amino acids include t-BuA.

"Aromatic Amino Acid" refers to a hydrophobic amino acid having a side chain containing at least one ring having a conjugated electron system (aromatic group). The aromatic group may be further substituted with groups such as alkyl, alkenyl, alkynyl, hydroxyl, sulfanyl, nitro and amino groups, as well as others. Examples of genetically encoded aromatic amino acids include Phe, Tyr and Trp. Commonly encountered non-genetically encoded aromatic amino acids include phenylglycine, 2-naphthylalanine, β-2-thienylalanine, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid, 4-chloro-phenylalanine, 2-fluorophenyl-alanine, 3-fluorophenylalanine and 4-fluorophenylalanine.

"Apolar Amino Acid" refers to a hydrophobic amino acid having a side chain that is generally uncharged at physiological pH and that is not polar. Examples of genetically encoded apolar amino acids include Gly, Pro and Met. Examples of non-encoded apolar amino acids include Cha.

"Aliphatic Amino Acid" refers to an apolar amino acid having a saturated or unsaturated straight chain, branched or cyclic hydrocarbon side chain. Examples of genetically encoded aliphatic amino acids include Ala, Leu, Val and Ile. Examples of non-encoded aliphatic amino acids include Nle.

"Hydrophilic Amino Acid" refers to an amino acid having a side chain that is attracted by aqueous solution. Examples of genetically encoded hydrophilic amino acids include Ser and Lys. Examples of non-encoded hydrophilic amino acids include Cit and hCys.

"Acidic Amino Acid" refers to a hydrophilic amino acid having a side chain pK value of less than 7. Acidic amino acids typically have negatively charged side chains at physiological pH due to loss of a hydrogen ion. Examples of genetically encoded acidic amino acids include Asp and Glu.

"Basic Amino Acid" refers to a hydrophilic amino acid having a side chain pK value of greater than 7. Basic amino acids typically have positively charged side chains at physiological pH due to association with hydronium ion. Examples of genetically encoded basic amino acids include Arg, Lys and His. Examples of non-genetically encoded basic amino acids include the non-cyclic amino acids ornithine, 2,3-diaminopropionic acid, 2,4-diaminobutyric acid and homoarginine.

"Polar Amino Acid" refers to a hydrophilic amino acid having a side chain that is uncharged at physiological pH, but which has a bond in which the pair of electrons shared in common by two atoms is held more closely by one of the atoms. Examples of genetically encoded polar amino acids include Asx and Glx. Examples of non-genetically encoded polar amino acids include citrulline, N-acetyl lysine and methionine sulfoxide.

"Cysteine-Like Amino Acid" refers to an amino acid having a side chain capable of forming a covalent linkage with a side chain of another amino acid residue, such as a disulfide linkage. Typically, cysteine-like amino acids generally have a side chain containing at least one thiol (SH) group. Examples of genetically encoded cysteine-like amino acids include Cys.

Examples of non-genetically encoded cysteine-like amino acids include homocysteine and penicillamine.

As will be appreciated by those having skill in the art, the above classification are not absolute -- several amino acids exhibit more than one characteristic property, and can therefore be included in more than one category. For example, tyrosine has both an aromatic ring and a polar hydroxyl group. Thus, tyrosine has dual properties and can be included in both the aromatic and polar categories. Similarly, in addition to being able to form disulfide linkages, cysteine also has apolar character. Thus, while not strictly classified as a hydrophobic or apolar amino acid, in many instances cysteine can be used to confer hydrophobicity to a peptide.

Certain commonly encountered amino acids which are not genetically encoded of which the peptides and peptide analogues of the invention may be composed include, but are not limited to, β-alanine (b-Ala) and other omega-amino acids such as 3-aminopropionic acid (Dap), 2,3-diaminopropionic acid (Dpr), 4-aminobutyric acid and so forth; α-aminoisobutyric acid (Aib); ε-aminohexanoic acid (Aha); δ-aminovaleric acid (Ava); N-methylglycine or sarcosine (MeGly); ornithine (Orn); citrulline (Cit); t-butylalanine (t-BuA); t-butylglycine (t-BuG); N-methylisoleucine (MeIle); phenylglycine (Phg); cyclohexylalanine (Cha); norleucine (Nle); 2-naphthylalanine (2-Nal); 4-chlorophenylalanine (Phe(4-Cl)); 2-fluorophenylalanine (Phe(2-F)); 3-fluorophenylalanine (Phe(3-F)); 4-fluorophenylalanine (Phe(4-F)); penicillamine (Pen); 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic); β-2-thienylalanine (Thi); methionine sulfoxide (MSO); homoarginine (hArg); N-acetyl lysine (AcLys); 2,3-diaminobutyric acid (Dab); 2,3-diaminobutyric acid (Dbu); p-aminophenylalanine (Phe(pNH_2)); N-methyl valine (MeVal); homocysteine (hCys) and homoserine (hSer). These amino acids also fall conveniently into the categories defined above.

The classifications of the above-described genetically encoded and non-encoded amino acids are summarized in **TABLE 1**, below. It is to be understood that **TABLE 1** is for illustrative purposes only and does not purport to be an exhaustive list of amino acid residues which may comprise the peptides and peptide analogues described herein. Other amino acid residues which are useful for making the peptides and peptide analogues described herein can be found, e.g., in Fasman, 1989, *CRC Practical Handbook of Biochemistry and Molecular Biology*, CRC Press, Inc., and the references cited therein. Amino acids not specifically mentioned herein can be conveniently classified into the above-described categories on the basis of known

behavior and/or their characteristic chemical and/or physical properties as compared with amino acids specifically identified.

TABLE 1

Classification	Genetically Encoded	Genetically Non-Encoded
Hydrophobic		
Aromatic	F, Y, W	Phg, Nal, Thi, Tic, Phe(4-Cl), Phe(2-F), Phe(3-F), Phe(4-F), Pyridyl Ala, Benzothienyl Ala
Apolar	M, G, P	
Aliphatic	A, V, L, I	t-BuA, t-BuG, MeIle, Nle, MeVal, Cha, bAla, MeGly, Aib
Hydrophilic		
Acidic	D, E	
Basic	H, K, R	Dpr, Orn, hArg, Phe(p-NH_2), DBU, A_2BU
Polar	Q, N, S, T, Y	Cit, AcLys, MSO, hSer
Cysteine-Like	C	Pen, hCys, p-methyl Cys

As used herein, a "detectable label" has the ordinary meaning in the art and refers to an atom (e.g., radionuclide), molecule (e.g., fluorescein), or complex, that is or can be used to detect (e.g., due to a physical or chemical property), indicate the presence of a molecule or to enable binding of another molecule to which it is covalently bound or otherwise associated. The term "label" also refers to covalently bound or otherwise associated molecules (e.g., a biomolecule such as an enzyme) that act on a substrate to produce a detectable atom, molecule or complex. Detectable labels suitable for use in the present invention include any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Labels useful in the present invention include biotin for staining with labeled streptavidin conjugate, magnetic beads (e.g., Dynabeads^{TM}), fluorescent dyes (e.g., fluorescein, Texas red, rhodamine, green fluorescent protein, enhanced green fluorescent protein, and the like), radiolabels (e.g., ^{3}H, ^{125}I, ^{35}S, ^{14}C, or ^{32}P), enzymes (e.g., hydrolases, particularly phosphatases such as alkaline phosphatase, esterases and glycosidases, or oxidoreductases, particularly peroxidases such as horseradish peroxidase, and others commonly used in ELISAs), substrates, cofactors, inhibitors, chemiluminescent groups, chromogenic agents, and colorimetric

labels such as colloidal gold or colored glass or plastic (e.g., polystyrene, polypropylene, latex, etc.) beads. Patents teaching the use of such labels include U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241. Means of detecting such labels are well known to those of skill in the art. Thus, for example, radiolabels and chemiluminescent labels may be detected using photographic film or scintillation counters, fluorescent markers may be detected using a photodetector to detect emitted light (e.g., as in fluorescence-activated cell sorting). Enzymatic labels are typically detected by providing the enzyme with a substrate and detecting the reaction product produced by the action of the enzyme on the substrate, and colorimetric labels are detected by simply visualizing the colored label. Thus, a label is any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. The label may be coupled directly or indirectly to the desired component of the assay according to methods well known in the art. Non-radioactive labels are often attached by indirect means. Generally, a ligand molecule (e.g., biotin) is covalently bound to the molecule. The ligand then binds to an anti-ligand (e.g., streptavidin) molecule which is either inherently detectable or covalently bound to a signal generating system, such as a detectable enzyme, a fluorescent compound, or a chemiluminescent compound. A number of ligands and anti-ligands can be used. Where a ligand has a natural anti-ligand, for example, biotin, thyroxine, and cortisol, it can be used in conjunction with the labeled, naturally occurring anti-ligands. Alternatively, any haptenic or antigenic compound can be used in combination with an antibody. The molecules can also be conjugated directly to signal generating compounds, e.g., by conjugation with an enzyme or fluorophore. Means of detecting labels are well known to those of skill in the art. Thus, for example, where the label is a radioactive label, means for detection include a scintillation counter, photographic film as in autoradiography, or storage phosphor imaging. Where the label is a fluorescent label, it may be detected by exciting the fluorochrome with the appropriate wavelength of light and detecting the resulting fluorescence. The fluorescence may be detected visually, by means of photographic film, by the use of electronic detectors such as charge coupled devices (CCDs) or photomultipliers and the like. Similarly, enzymatic labels may be detected by providing the appropriate substrates for the enzyme and detecting the resulting reaction product. Also, simple colorimetric labels may be detected by observing the color associated with the label. It will be

appreciated that when pairs of fluorophores are used in an assay, it is often preferred that they have distinct emission patterns (wavelengths) so that they can be easily distinguished.

As used herein, the term "substantially identical" in the context of comparing amino acid sequences, means that the sequences have at least about 70%, at least about 80%, or at least about 90% amino acid residue identity when compared and aligned for maximum correspondence. An algorithm that is suitable for determining percent sequence identity and sequence similarity is the FASTA algorithm, which is described in Pearson, W.R. & Lipman, D.J., 1988, *Proc. Natl. Acad. Sci. U.S.A.* 85: 2444. See also W. R. Pearson, 1996, *Methods Enzymol.* 266: 227-258. Preferred parameters used in a FASTA alignment of DNA sequences to calculate percent identity are optimized, BL50 Matrix 15: -5, k-tuple = 2; joining penalty = 40, optimization = 28; gap penalty -12, gap length penalty =-2; and width = 16.

As used herein, the terms "test compound" or "test agent" are used interchangeably and refer to a candidate agent that may have enhancer/agonist, or inhibitor/antagonist activity, e.g., inhibiting or enhancing an interaction such as PDZ-PL binding. The candidate agents or test compounds may be any of a large variety of compounds, both naturally occurring and synthetic, organic and inorganic, and including polymers (e.g., oligopeptides, polypeptides, oligonucleotides, and polynucleotides), small molecules, antibodies (as broadly defined herein), sugars, fatty acids, nucleotides and nucleotide analogs, analogs of naturally occurring structures (e.g., peptide mimetics, nucleic acid analogs, and the like), and numerous other compounds. In certain embodiment, test agents are prepared from diversity libraries, such as random or combinatorial peptide or non-peptide libraries. Many libraries are known in the art that can be used, *e.g.*, chemically synthesized libraries, recombinant (*e.g.*, phage display libraries), and *in vitro* translation-based libraries. Examples of chemically synthesized libraries are described in Fodor et al., 1991, *Science* 251:767-773; Houghten et al., 1991, *Nature* 354:84-86; Lam et al., 1991, *Nature* 354:82-84; Medynski, 1994, *Bio/Technology* 12:709-710; Gallop et al., 1994, J. *Medicinal Chemistry* 37(9):1233-1251; Ohlmeyer et al., 1993, *Proc. Natl. Acad. Sci. USA* 90:10922-10926; Erb et al., 1994, *Proc. Natl. Acad. Sci. USA* 91:11422-11426; Houghten et al., 1992, *Biotechniques* 13:412; Jayawickreme et al., 1994, *Proc. Natl. Acad. Sci. USA* 91:1614-1618; Salmon et al., 1993, *Proc. Natl. Acad. Sci. USA* 90:11708-11712; PCT Publication No. WO 93/20242; and Brenner and Lerner, 1992, *Proc. Natl. Acad. Sci. USA* 89:5381-5383. Examples of phage display libraries are described in Scott and Smith, 1990,

Science 249:386-390; Devlin et al., 1990, *Science*, 249:404-406; Christian, R.B., et al., 1992, *J. Mol. Biol.* 227:711-718); Lenstra, 1992, *J. Immunol. Meth.* 152:149-157; Kay et al., 1993, *Gene* 128:59-65; and PCT Publication No. WO 94/18318 dated August 18, 1994. *In vitro* translation-based libraries include but are not limited to those described in PCT Publication No. WO 91/05058 dated April 18, 1991; and Mattheakis et al., 1994, *Proc. Natl. Acad. Sci. USA* 91:9022-9026. By way of examples of nonpeptide libraries, a benzodiazepine library (see *e.g.*, Bunin et al., 1994, *Proc. Natl. Acad. Sci. USA* 91:4708-4712) can be adapted for use. Peptoid libraries (Simon et al., 1992, *Proc. Natl. Acad. Sci. USA* 89:9367-9371) can also be used. Another example of a library that can be used, in which the amide functionalities in peptides have been permethylated to generate a chemically transformed combinatorial library, is described by Ostresh et al. (1994, *Proc. Natl. Acad. Sci. USA* 91:11138-11142).

The term "specific binding" refers to binding between two molecules, for example, a ligand and a receptor, characterized by the ability of a molecule (ligand) to associate with another specific molecule (receptor) even in the presence of a plurality of other diverse molecules, i.e., to show preferential binding of one molecule for another in a heterogeneous mixture of molecules. Specific binding of a ligand to a receptor is also evidenced by reduced binding of a detectably labeled ligand to the receptor in the presence of excess unlabeled ligand (i.e., a binding competition assay).

As used herein, a "plurality" of PDZ proteins (or corresponding PDZ domains or PDZ fusion polypeptides) has its usual meaning. In some embodiments, (Susan our US associate has cautioned us to remove phrases such as "in one embodiment" as the applicant may be required to include all of these features as limitations in the claims. I am not sure if you agree, it is now our practice to use the phrase "in certain embodiments" OK with me the plurality is at least 5, and often at least 25, at least 40, or at least 60 different PDZ proteins. In some embodiments, the plurality is selected from the list of PDZ polypeptides listed in **TABLE 5.** In some embodiments, the plurality of different PDZ proteins are from (i.e., expressed in) a particular specified tissue or a particular class or type of cell. In some embodiments, the plurality of different PDZ proteins represents a substantial fraction (e.g., typically at least 50%, more often at least 80%) of all of the PDZ proteins known to be, or suspected of being, expressed in the tissue or cell(s), e.g., all of the PDZ proteins known to be present in neurons. In some

embodiments, the plurality is at least 50%, usually at least 80%, at least 90% or all of the PDZ proteins disclosed herein as being expressed in a particular cell.

When referring to PL peptides (or the corresponding proteins, e.g., corresponding to those listed in **TABLE 2**, or elsewhere herein) a "plurality" may refer to at least 5, at least 10, and often at least 25 PLs such as those specifically listed herein, or to the classes and percentages set forth *supra* for PDZ domains.

The term "cytodestructive disorder" generally refers to a disorder in which an injury to a tissue is sustained that results in the destruction or dysfunction of cells. Specific examples of such disorders include, but are not limited to, tissue ischemia, thermal burns, electrical burns, burns sustained from contact with caustic solutions, poisonings, endocrine and metabolic derangements, nutritional deficiencies, exposure to solar or ionizing radiation, malignant transformation, and mechanical tissue deformation such as might occur in tissue trauma.

The term "neurological disorder," "neurological injury", "neurological disease" and other related terms generally refers to a disorder correlated with some type neuronal insult or neuronal cell death. Specific examples of such disorders include, but are not limited to, stroke, ischemic stroke, glaucoma, retinal ischemia, Parkinson's disease, Huntington's disease, Alzheimer's disease, epilepsy, inherited ataxias and motor neuron diseases. Further examples of such disorders include, but are not limited to, disorders of synaptic transmission in the brain, such disorders resulting in impairments of learning, memory, neuropsychatric and mood disorders, and congenital disorders of mentation.

A "stroke" has the meaning normally accepted in the art and generally refers to neurological injury resulting from impaired blood flow regardless of cause. Potential causes include, but are not limited to, embolism, hemorrhage and thrombosis. An "ischemic stroke" refers more specifically to a type of stroke that is of limited extent and caused due to blockage of blood flow.

A difference in general is typically considered to be "statistically significant" if the difference is less than experimental error. Thus a difference is considered statistically significant if the probability of the observed difference occurring by chance (the p-value) is less than some predetermined level. As used herein a "statistically significant difference" can refer to a p-value that is < 0.05, preferably < 0.01 and most preferably < 0.001.

II. General

The present inventors have identified interactions between PDZ proteins and proteins that contain a PL motif that are involved in various biological functions in different types of cells. Some of these interactions involve PDZ:PL protein interactions between proteins that have important roles in neuronal cells. As such, modulation of these interactions has direct implications for the treatment of various disorders of mammalian cell injury, including, but not limited to, neurological disorders such as stroke and brain ischemia.

The inventors have identified distinct strategies for treating various neurological disorders based on PDZ:PL interations. One strategy is based upon the finding that disrupting the activity of a member of the TRP cation channel family, is protective for cellular damage induced by OGD (oxygen-glucose deprivation) or anoxia. The inventors have determined common structural features of a class of polypeptides that are effective in disrupting the interaction between a member of the TRP cation channel family and PDZ domain-containing polypeptides. These polypeptides are thus useful in treating disorders associated with OGD. The second strategy is based upon the recognition that several types of ion channels can be grouped and regulated by PDZ proteins, and that disruption of these interactions can modulate the structure of these complexes and provide protection against damage resulting from OGD.

The current inventors have thus identified compounds that inhibit the interactions between these different proteins, as well as developed methods for designing additional compounds. One general class of inhibitors is those that mimic the carboxy terminus of a PL protein and thus interfere with the ability of the carboxy terminus of the PL protein to bind its cognate PDZ protein. Another general class of inhibitors includes the PDZ domain from a PDZ protein that is involved in an interaction that is to be disrupted. These inhibitors bind the PL protein that is the cognate ligand for the PDZ protein of interest and thus prevent binding between the PL protein and PDZ protein. Because the PDZ:PL protein interactions that are described herein are involved in the biological activity of mammalian, and especially neuronal cells, the inhibitors that are provided can be used to inhibit PDZ:PL protein interactions for the treatment of neurological disorders such as stroke, ischemia, Parkinson's disease, Huntington's disease, Alzheimer's disease, epilepsy, inherited ataxias, motor neuron diseases as well as myocardial ischemia, retinal ischemia and glaucoma. Methods for determining whether a test

compound acts a modulator of a particular PDZ protein and PL protein binding pair are also described.

For those PDZ proteins containing multiple PDZ domains, the methods that are provided can be utilized to determine to which specific domain(s) a particular PL protein of interest binds. The methods can thus be utilized to identify or design inhibitors that have increased selectivity for a particular PDZ domain. The methods that are disclosed can also be used to identify inhibitors with high binding affinity. Because TRP channels play a key regulatory role in many cell types, an initial set of studies were undertaken to determine which PDZ proteins bind to the PL of TRPM7. These analyses were conducted using the A and G assays described in detail below. Without intending to be limiting, the PDZ proteins identified as being able to bind TRPM7 are listed in **TABLE 6.**

The C-terminal sequences of the various TRP channels that contain a PL sequence are listed in **TABLE 4.** Because the C-terminal region of the PL protein is the most common region that binds to PDZ proteins, agents that include similar amino acid motifs can be used to inhibit binding between TRP proteins and the PDZ proteins that bind to them. As described in greater detail below, for example, certain classes of peptide inhibitors typically include at least 2 contiguous amino acids from the C-terminus of the TRP proteins listed in **TABLE 4,** but can include 3-20 or more contiguous amino acids from the C-terminus.

Additional studies identify the structural motifs common to the polypeptides capable of inhibiting the interaction between TRPM7 and PDZ domains. One class of compounds are polypeptides that have the following characteristics: 1) a length of about 3-20 amino acids (although somewhat longer polypeptides can be used), and 2) a C-terminal consensus sequence of X-L/I/V-X-V/L/A (the slash separates different amino acids that can appear at a given position). These polypeptides also typically have IC_{50} values of less than 50μM.

The inventors have also found that the C-termini of several MAGUK (membrane associated Guanylate Kinases) are themselves PL sequences and thus can bind PDZ proteins. Accordingly, another class of inhibitors are those that disrupt binding between the PL sequences of these PDZ proteins and their PDZ binding partners. These proteins are involved in organizing signaling complexes associated with excitotoxic, anoxic or cytodestructive cellular death.

Interactions of this type thus provide another therapeutic target for treatment of various neurological diseases.

Although the foregoing classes of inhibitors are based upon the C-terminal sequences of PL proteins that bind a PDZ protein, as alluded to above, another class of inhibitors includes polypeptides that include all or a part of a PDZ domain that binds to the PL sequence of a TRP channel protein or TRP associated protein. Because inhibitors in this class typically include most or the entire PDZ domain, polypeptide inhibitors in this class typically are at least 50-70 amino acids in length.

The various classes of polypeptide inhibitors just described can also be fusion proteins. These generally include a PL inhibitor peptide sequence such as those just listed that is fused to another sequence that encodes a separate protein domain. One specific example of an inhibitory fusion protein is one in which a PL sequence (e.g., it might simply be easier to reinsert the list cite Table 10 and Table 11) is coupled to a transmembrane transporter peptide. As described in greater detail infra, a variety of different transmembrane transporter peptides can be utilized.

Although certain classes of inhibitors such as those just described are polypeptides, other inhibitors are peptide mimetics or variants of these polypeptides as described in greater detail infra. Regardless of type, the inhibitors typically had IC_{50} values less than 50 μM, 25 μM, 10 μM, 0.1 μM or 0.01 μM. In general the inhibitors typically have an IC_{50} value of between 0.1 - 1 μM. These inhibitors can be formulated as pharmaceutical compositions and then used in the treatment of various neurological disorders such as those listed above.

The following sections provide additional details regarding the identification of PDZ:PL interactions in neuron cells, the structural characteristics of inhibitors that disrupt these interactions and treatment methods utilizing such inhibitors.

III. Identification of Candidate PL Proteins and Synthesis of Peptides

A PL protein (short for PDZ Ligand protein), such as the TRP proteins described herein, is a protein (or a C-terminal fragment thereof) that can bind PDZ proteins via its carboxy terminus. PDZ proteins, in turn, are proteins with PDZ domains, which are domains common to three prototypical proteins: post synaptic density protein -95 (PSD-95), Drosophila large disc protein and Zonula Occludin 1 protein (see, e.g., Gomperts et al., 1996, *Cell* 84:659-662; see

also, Songyang et al., 1997, *Science* 275:73; and Doyle et al., 1996, *Cell* 88:1067-1076). Certain classes of PDZ proteins contain three PDZ domains, one SH3 domain and one guanylate kinase domain. As described in greater detail herein, PL proteins have certain carboxy terminal motifs that enable these proteins to functions as ligands to PDZ proteins. When these carboxy terminal regions are referred to, the positioning of the carboxy terminal residues are sometimes referred to herein by a numbered position, which is illustrated in the following scheme:

Position: -3 -2 -1 0 (C- terminal)

Certain PDZ domains are bound by the C-terminal residues of PDZ-binding proteins. To identify TRP channels and TRP associated proteins containing a PL motif, the C-terminal residues of sequences were visually inspected to identify sequences that bind to PDZ-domain containing proteins (see, e.g., Doyle et al., 1996, *Cell* 85, 1067; Songyang et al., 1997, *Science* 275, 73). **TABLES 2 and 4** list these proteins, and provide corresponding C-terminal sequences and GenBank accession numbers.

TABLE 2: Glutamate Receptors with PL Sequences

Name	GI#	PL	internal PL ID
NMDAR1	307302	X	AA216
NMDAR1-1	292282	X	AA216
NMDAR1-4	472845	X	AA216
NMDAR1-3b	2343286	X	AA216
NMDAR1-4b	2343288	X	AA216
NMDAR1-2	11038634		
NMDAR1-3	11038636		
NMDAR2C	6006004	X	AA180
NMDAR3	560546	X	AA34.1
NMDAR3A	17530176		
NMDAR2B	4099612	X	
NMDAR2A	558748	X	AA34.2
NMDAR2D	4504130	X	
GluR2	3287973	X	
GluR3	481504	X	
GluR1	1169961	X	
GluR5	729597	X	
GluR6	2492627	X	

GluR7	12729188	X	

A. Preparation of Peptides

1) Chemical Synthesis

Technique for the preparation of peptides and peptide analogues of the current invention are well known in the art. For example, the peptides may be prepared in linear form using conventional solution or solid phase peptide syntheses and cleaved from the resin followed by purification procedures (Creighton, 1983, *Protein Structures And Molecular Principles*, W.H. Freeman and Co., N.Y.). Suitable procedures for synthesizing the peptides described herein are well known in the art. The composition of the synthetic peptides may be confirmed by amino acid analysis or sequencing (e.g., the Edman degradation procedure and mass spectroscopy).

In addition, analogues and derivatives of the peptides can be chemically synthesized. The linkage between each amino acid of the peptides of the invention may be an amide, a substituted amide or an isostere of amide. Nonclassical amino acids or chemical amino acid analogues can be introduced as a substitution or addition into the sequence. Non-classical amino acids include, but are not limited to, the D-isomers of the common amino acids, α-amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid, γ-Abu, ε-Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β-alanine, fluoro-amino acids, designer amino acids such as β-methyl amino acids, Cα-methyl amino acids, Nα-methyl amino acids, and amino acid analogues in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

Synthetic peptides of defined sequence (e.g., corresponding to the carboxyl-termini of the indicated proteins) can be synthesized by any standard resin-based method (see, e.g., U. S. Patent No. 4,108,846; see also, Caruthers et al., 1980, *Nucleic Acids Res. Symp. Ser.*, 215-223; Horn et al., 1980, *Nucleic Acids Res. Symp. Ser.*, 225-232; Roberge, et al., 1995, *Science* 269:202). The peptides used in the assays described herein were prepared by the FMOC (see, e.g., Guy and Fields, 1997, *Meth. Enz.* 289:67-83; Wellings and Atherton, 1997, *Meth. Enz.*289:44-67). In some cases (e.g., for use in the A and G assays of the invention), peptides were labeled with biotin at the amino-terminus by reaction with a four-fold excess of biotin

methyl ester in dimethylsulfoxide with a catalytic amount of base. The peptides were cleaved from the resin using a halide containing acid (e.g. trifluoroacetic acid) in the presence of appropriate antioxidants (e.g. ethanedithiol) and excess solvent lyophilized.

2) Recombinant Synthesis

If the peptide is composed entirely of gene-encoded amino acids, or a portion of it is so composed, the peptide or the relevant portion may also be synthesized using conventional recombinant genetic engineering techniques. For recombinant production, a polynucleotide sequence encoding a linear form of the peptide is inserted into an appropriate expression vehicle, *i.e.*, a vector which contains the necessary elements for the transcription and translation of the inserted coding sequence, or in the case of an RNA viral vector, the necessary elements for replication and translation. The expression vehicle is then transfected into a suitable target cell which will express the peptide. Depending on the expression system used, the expressed peptide is then isolated by procedures well-established in the art. Methods for recombinant protein and peptide production are well known in the art (see, *e.g.*, Maniatis et al., 1989, *Molecular Cloning A Laboratory Manual*, Cold Spring Harbor Laboratory, N.Y.; and Ausubel et al., 1989, *Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley Interscience, N.Y.).

A variety of host-expression vector systems may be utilized to express the peptides described herein. These include, but are not limited to, microorganisms such as bacteria transformed with recombinant bacteriophage DNA or plasmid DNA expression vectors containing an appropriate coding sequence; yeast or filamentous fungi transformed with recombinant yeast or fungi expression vectors containing an appropriate coding sequence; insect cell systems infected with recombinant virus expression vectors (*e.g.*, baculovirus) containing an appropriate coding sequence; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus or tobacco mosaic virus) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing an appropriate coding sequence; or animal cell systems.

The expression elements of the expression systems vary in their strength and specificities. Depending on the host/vector system utilized, any of a number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used in the expression vector. For example, when cloning in bacterial systems, inducible

promoters such as pL of bacteriophage λ, plac, ptrp, ptac (ptrp-lac hybrid promoter) and the like may be used; when cloning in insect cell systems, promoters such as the baculovirus polyhedron promoter may be used; when cloning in plant cell systems, promoters derived from the genome of plant cells (*e.g.*, heat shock promoters; the promoter for the small subunit of RUBISCO; the promoter for the chlorophyll a/b binding protein) or from plant viruses (*e.g.*, the 35S RNA promoter of CaMV; the coat protein promoter of TMV) may be used; when cloning in mammalian cell systems, promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the adenovirus late promoter; the vaccinia virus 7.5 K promoter) may be used; when generating cell lines that contain multiple copies of expression product, SV40-, BPV- and EBV-based vectors may be used with an appropriate selectable marker.

In cases where plant expression vectors are used, the expression of sequences encoding the peptides of the invention may be driven by any of a number of promoters. For example, viral promoters such as the 35S RNA and 19S RNA promoters of CaMV (Brisson et al., 1984, Nature 310:511-514), or the coat protein promoter of TMV (Takamatsu et al., 1987, EMBO J. 6:307-311) may be used; alternatively, plant promoters such as the small subunit of RUBISCO (Coruzzi et al., 1984, EMBO J. 3:1671-1680; Broglie et al., 1984, Science 224:838-843) or heat shock promoters, *e.g.*, soybean hsp17.5-E or hsp17.3-B (Gurley et al., 1986, Mol. Cell. Biol. 6:559-565) may be used. These constructs can be introduced into planleukocytes using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, microinjection, electroporation, etc. For reviews of such techniques see, *e.g.*, Weissbach & Weissbach, 1988, *Methods for Plant Molecular Biology*, Academic Press, NY, Section VIII, pp. 421-463; and Grierson & Corey, 1988, *Plant Molecular Biology*, 2d Ed., Blackie, London, Ch. 7-9.

In one insect expression system that may be used to produce the peptides of the invention, *Autographa californica* nuclear polyhidrosis virus (AcNPV) is used as a vector to express the foreign genes. The virus grows in *Spodoptera frugiperda* cells. A coding sequence may be cloned into non-essential regions (for example the polyhedron gene) of the virus and placed under control of an AcNPV promoter (for example, the polyhedron promoter). Successful insertion of a coding sequence will result in inactivation of the polyhedron gene and production of non-occluded recombinant virus (*i.e.*, virus lacking the proteinaceous coat coded for by the polyhedron gene). These recombinant viruses are then used to infect *Spodoptera*

frugiperda cells in which the inserted gene is expressed. (*e.g.*, see Smith *et al.*, 1983, J. Virol. 46:584; Smith, U.S. Patent No. 4,215,051). Further examples of this expression system may be found in *Current Protocols in Molecular Biology,* Vol. 2, Ausubel *et al.*, eds., Greene Publish. Assoc. & Wiley Interscience.

In mammalian host cells, a number of viral based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, a coding sequence may be ligated to an adenovirus transcription/translation control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region E1 or E3) will result in a recombinant virus that is viable and capable of expressing peptide in infected hosts. (*e.g.*, See Logan & Shenk, 1984, Proc. Natl. Acad. Sci. USA 81:3655-3659). Alternatively, the vaccinia 7.5 K promoter may be used, (see, *e.g.*, Mackett et al., 1982, Proc. Natl. Acad. Sci. USA 79:7415-7419; Mackett et al., 1984, J. Virol. 49:857-864; Panicali et al., 1982, Proc. Natl. Acad. Sci. USA 79:4927-4931).

Other expression systems for producing linear peptides of the invention will be apparent to those having skill in the art.

B. Purification of Peptides and Peptide Analogues

The peptides and peptide analogues of the invention can be purified by art-known techniques such as high performance liquid chromatography, ion exchange chromatography, gel electrophoresis, affinity chromatography and the like. The actual conditions used to purify a particular peptide or analogue will depend, in part, on factors such as net charge, hydrophobicity, hydrophilicity, etc., and will be apparent to those having skill in the art. The purified peptides can be identified by assays based on their physical or functional properties, including radioactive labeling followed by gel electrophoresis, radioimmuno-assays, ELISA, bioassays, and the like.

For affinity chromatography purification, any antibody which specifically binds the peptides or peptide analogues may be used. For the production of antibodies, various host animals, including but not limited to rabbits, mice, rats, etc., may be immunized by injection with a peptide. The peptide may be attached to a suitable carrier, such as BSA or KLH, by means of a side chain functional group or linkers attached to a side chain functional group. Various adjuvants may be used to increase the immunological response, depending on the host

species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacilli Calmette-Guerin) and *Corynebacterium parvum.*

Monoclonal antibodies to a peptide may be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Koehler and Milstein, 1975, Nature 256:495-497, the human B-cell hybridoma technique, Kosbor et al., 1983, Immunology Today 4:72; Cote et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:2026-2030 and the EBV-hybridoma technique (Cole et al., 1985, *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96 (1985)). In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:6851-6855; Neuberger et al., 1984, Nature 312:604-608; Takeda et al., 1985, Nature 314:452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. Alternatively, techniques described for the production of single chain antibodies (U.S. Patent No. 4,946,778) can be adapted to produce peptide-specific single chain antibodies.

Antibody fragments which contain deletions of specific binding sites may be generated by known techniques. For example, such fragments include but are not limited to $F(ab')_2$ fragments, which can be produced by pepsin digestion of the antibody molecule and Fab fragments, which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragments. Alternatively, Fab expression libraries may be constructed (Huse et al., 1989, Science 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for the peptide of interest.

The antibody or antibody fragment specific for the desired peptide can be attached, for example, to agarose, and the antibody-agarose complex is used in immunochromatography to purify peptides of the invention. *See*, Scopes, 1984, *Protein Purification: Principles and Practice*, Springer-Verlag New York, Inc., NY, Livingstone, 1974, Methods Enzymology: Immunoaffinity Chromatography of Proteins 34:723-731.

For the peptides used in the present invention, cleavage from resin and lyophilization was followed by peptides being redissolved and purified by reverse phase high

performance liquid chromatography (HPLC). One appropriate HPLC solvent system involves a Vydac C-18 semi-preparative column running at 5 ml per minute with increasing quantities of acetonitrile plus 0.1% trifluoroacetic acid in a base solvent of water plus 0.1% trifluoroacetic acid. After HPLC purification, the identities of the peptides are confirmed by MALDI cation-mode mass spectrometry. As noted, exemplary biotinylated peptides are provided in **TABLE 2**.

IV. TRP channels A. NOMENCLATURE (Taken from C. Montell, et al. A unified nomenclature for the superfamily of TRP cation channels. *Mol.Cell* 9,(2):229-231, 2002.)

The TRP superfamily includes a diversity of non-voltage-gated cation channels that vary significantly in their selectivity and mode of activation. Nevertheless, members of the TRP superfamily share significant sequence homology and predicted structural similarities. Until recently most of the genes and proteins that comprise the TRP superfamily have had multiple names and, in at least one instance, two distinct genes belonging to separate subfamilies have the same name. Moreover, there are many cases in which highly related proteins that belong to the same subfamily have unrelated names. Therefore, to minimize confusion, a unified nomenclature has been accepted for the TRP superfamily.

The unified TRP nomenclature focuses on three subfamilies (TRPC, TRPV, and TRPM) that bear significant similarities to the founding member of this superfamily, Drosophila TRP, and which include highly related members in worms, flies, mice, and humans (**TABLE 3**). Members of the three subfamilies contain six transmembrane segments, a pore loop separating the final two transmembrane segments, and similarity in the lengths of the cytoplasmic and extracellular loops. In addition, the charged residues in the S4 segment that appear to contribute to the voltage sensor in voltage-gated ion channels are not conserved. The TRP-Canonical (TRPC) subfamily (formerly short-TRPs or STRPs) is comprised of those proteins that are the most highly related to Drosophila TRP. The TRPV subfamily (formerly OTRPC), is so named based on the original designation, Vanilloid Receptor 1 (VR1), for the first mammalian member of this subfamily (now TRPV1). The name for the TRPM subfamily (formerly long-TRPs or LTRPs) is derived from the first letter of Melastatin, the former name (now TRPM1) of the founding member of this third subfamily of TRP-related proteins. Based on amino acid

homologies, the mammalian members of these three subfamilies can be subdivided into several groups each (**TABLE 4** and **Figure 1**).

Table 3. Number of TRP Genes in Worms (C. elegans), Flies (Drosophila melanogaster), Mice, and Humans

Subfamily	Worms	Flies	Mice	Humans
TRPC	**3**	**3**	**7**	**6[a]**
TRPV	**5**	**2**	**5**	**5**
TRPM	**4**	**1**	**8**	**8**

[a]TRPC2 is a pseudogene and is not counted.

TABLE 4. Nomenclature of the Mammalian TRP Superfamily

Name	Group	Former Names	Accession Numbers
TRPC Subfamily			
TRPC1	1	TRP1 TRPC1	(CAA61447) , (AAA93252)
TRPC2	2	TRP2 TRPC2	(X89067) , (AAD17195) , (AAD17196) , (AAG29950) , (AAG29951) , (AAD31453) , (CAA06964)
TRPC3	3	TRP3 TRPC3	(AAC51653)
TRPC4	4	TRP4 TRPC4	(CAA68125) , (BAA23599)
TRPC5	4	TRP5 TRPC5	(AAC13550) , (CAA06911) , (CAA06912)
TRPC6	3	TRP6 TRPC6	NP_038866
TRPC7	3	TRP7 TRPC7	(AAD42069) , NP_065122
TRPV Subfamily			
TRPV1	1	VR1 OTRPC1	(AAC53398)
TRPV2	1	VRL-1 OTRPC2 GRC	(AAD26363) , (AAD26364) , (BAA78478)
TRPV3 (not assigned)			
TRPV4	2	OTRPC4 VR-OAC TRP12 VRL-2	(AAG17543) , (AAG16127) , (AAG28027) , (AAG28028) , (AAG28029) , (CAC20703)
TRPV5	3	ECaC1 CaT2	(CAB40138)
TRPV6	3	CaT1 ECaC2 CaT-L	(AAD47636) (CAC20416) (CAC20417)

TRPM Subfamily			
TRPM1	1	Melastatin	(AAC13683) , (AAC80000)
TRPM2	2	TRPC7 LTRPC2	(BAA34700)
TRPM3	1	KIAA1616 LTRPC3	(AA038185)
TRPM4	3	TRPM4 LTRPC4	(H18835)
TRPM5	3	MTR1 LTRPC5	(AAF26288)
TRPM6	4	Chak2	(AF350881)
TRPM7	4	TRP-PLIK Chak1 LTRPC7	(AAF73131), (AY032951)
TRPM8	2	TRP-p8	(AC005538)

The numbering system for the mammalian TRPC, TRPV, and TRPM proteins takes into account the order of their discovery and, in as many cases as possible, the number that has already been assigned to the genes and proteins (**TABLE 4**). In the case of the TRPV proteins, the numbering system is also based in part on the groupings of the TRPV proteins. New members of each subfamily will maintain the same root name and, with the exception of TRPV3, will be assigned the next number in the sequence. Currently, TRPV3 is unassigned to maintain the TRPV1/ TRPV2 and TRPV5/TRPV6 groupings and so that the former OTRPC4 could be renamed TRPV4. The next TRPV protein will be designated TRPV3.

B. Current Knowledge on TRP channel function. (Taken directly from Clapham (2003) Nature 426:517 – 524,

Transient receptor potential (TRP) channels were first described in Drosophila, where photoreceptors carrying trp gene mutations exhibited a transient voltage response to continuous light. They have been called store-operated channels (SOCs), but this description is theoretical and related to a poorly understood phenomenon. All TRP channels are putative six-transmembrane (6TM) polypeptide subunits that assemble as tetramers to form cation-permeable pores (Figure 2). In general, they are almost ubiquitously expressed and most have splice variants. So most cells have a number of TRP channel proteins.

Figure 2 emphasizes the diversity of TRP cytoplasmic domains.

The selectivity filter (light blue and inset) is formed by amino acids that dip into the bilayer (pore loops), one contributed from each of the four subunits. S5 has been removed to emphasize the link between the S6 gating helix and the TRP C-terminal polypeptide chain. The TRP box is EWKFAR (SEQ ID NO:2) in TRPC, but is less conserved in TRPV and TRPM. CC indicates a coiled-coil domain. Ankyrin repeats (AnkR) range from 0 to 14 in number (3 or 4 in TRPV and TRPC, 14 in ANKTM; not shown). Numbers on the right indicate range in length. CIRB, putative calmodulin- and IP3R-binding domain; EF hand, canonical helix–loop–helix Ca^{2+}-binding domain; PDZ, amino acids binding PDZ domains; PLIK, phospholipase-C-interacting kinase, an atypical protein kinase intrinsic to the TRPM6 and TRPM7 polypeptide chains; Nudix, NUDT9 hydrolase protein homologue binding ADP ribose.

The TRPC (canonical TRP) subfamily

All mammalian TRPC proteins appear to be analogous to the TRP involved in Drosophila phototransduction, in that they function as receptor-operated channels. They are activated by stimulation of G-protein-coupled receptors (GPCRs) and receptor tyrosine kinases.

TRPC1, the first mammalian TRP reported, forms heteromeric channels with TRPC4 and/or TRPC5. The properties of the heteromultimers are distinct from those of TRPC4 and TRPC5 homomultimers. TRPC5, but not TRPC1, is present in hippocampal growth cones and modulates neurite extension. Mice lacking TRPC4 have defects in agonist-induced vasoregulation and lung microvascular permeability.

TRPC3, TRPC6 and TRPC7 proteins share 75% identity, have relatively low selectivity for Ca^{2+} over Na^{+}, and are sensitive to the intracellular concentration of Ca^{2+} ($[Ca^{2+}]i$). Diacylglycerol (DAG) analogs potentiate their activity, but not through protein kinase C activation. TRPC3 has been investigated extensively as a putative inositol-1,4,5-trisphosphate (InsP3) receptor (IP3R)-binding SOC, with conflicting results. All of the TPRC3, TRPC6 and TRPC7 subfamily are highly expressed in smooth and cardiac muscle cells, making them candidates for the receptor-activated nonselective cation channels known to exist in these sites. In support of this idea, TRPC6 is an essential part of the 1-adrenoreceptor-activated cation channel in rabbit portal vein myocytes. They may also have roles in the regulation of vascular tone, airway resistance and cardiac function. TRPC2 appears to be a pseudogene in humans, but its rat orthologue encodes an important sensor localized to neuronal microvilli in the

its rat orthologue encodes an important sensor localized to neuronal microvilli in the vomeronasal organ. Trpc2-deficient mice display abnormal mating behavior, consistent with a role for this channel in pheromone signaling.

The TRPV (vanilloid receptor, osm9-like) subfamily

TRPV1 was identified by expression cloning using the 'hot' pepper-derived vanilloid compound capsaicin as a ligand. TRPV1 is a Ca^{2+}-permeant channel that is potentiated by heat (>43 °C) and decreased pH, and inhibited by intracellular phosphatidylinositol-4,5-bisphosphate (PIP2). Its thermal sensitivity is enhanced by bradykinin and nerve growth factor, which appear to act via phospholipase C (PLC) to hydrolyse PIP2, releasing inhibition of the channel. Trpv1-/- mice are defective in nociceptive, inflammatory and hypothermic responses to vanilloid compounds, supporting the interpretation that TRPV1 contributes to acute thermal nociception and hyperalgesia after tissue injury. TRPV1 also participates in mechanically evoked purinergic signaling by the bladder urothelium.

TRPV2, which is 50% identical to TRPV1, may mediate high-threshold (>52 °C) noxious heat sensation, perhaps through lightly myelinated A nociceptors. Interestingly, TRPV2 translocates from intracellular pools upon insulin growth factor stimulation of transfected cells. Stretch reportedly increases TRPV2 translocation, and cardiac-specific transgene expression of TRPV2 results in Ca^{2+}-overload-induced cardiomyopathy. But it is not surprising that overexpression of a Ca^{2+}-permeant channel induces cardiomyopathy, because such TRP channels are deleterious to many cells, including neurons. In fact, the mechanism of pain-relieving topical capsaicin is due, in part, to neuronal cell death.

Increased temperature also activates TRPV3 (>31 °C) and TRPV4 (>25 °C). The neuronal distribution of TRPV3 overlaps with TRPV1, raising the interesting possibility that they may heteromultimerize. TRPV3 is also highly expressed in skin, tongue and the nervous system, possibly explaining the activity of 'warm-sensitive' neurons. The effect of temperature on rates of biological processes is expressed as the 10 °C temperature coefficient1: Q10 = rate(T + 10 °C)/rate(T). Most ion channels and enzymes have gating Q10 values of 3–5, but the Q10 of TRPV3 gating is >20, and for TRPV1 and TRPV4 it is estimated to be 10–20. TRPV4 current is potentiated by hypotonicity (cell swelling). Trpv4-/- mice have a marginally impaired renal response to hypertonicity, probably due to abnormal central control of antidiuretic hormone

secretion. Hypotonicity increases TRPV4-mediated current in primary afferent nociceptive nerve fibers, an effect that is enhanced by the hyperalgesic inflammatory mediator prostaglandin E2. Expressed TRPV4 may be gated by epoxyeicosatrienoic acids.

TRPV5 and TRPV6 comprise a subfamily of homomeric and heteromeric channels found in transporting epithelia of the kidney and intestine. They show strong inwardly rectifying currents and are the most Ca^{2+}-selective TRP channels (permeability ratio PCa/PNa > 100), suggesting that they mediate Ca^{2+} uptake. Both are inactivated by $[Ca^{2+}]I$; TRPV6 shows voltage-dependent intracellular Mg^{2+} blockade.

The TRPM (melastatin) subfamily and TRPA

TRPM1 (melastatin) was initially identified as a transcript that showed decreased expression in highly metastatic versus non-metastatic melanoma cells.

TRPM2 forms a Ca^{2+}-permeant channel that is gated by binding of ADP ribose (EC_{50} 100 μM) and nicotinamide adenine dinucleotide (NAD; 1 mM) to a carboxy-terminal NUDT9 Nudix hydrolase domain. ADP ribose is a breakdown product of NAD, CD38, cyclic ADP ribose (a Ca^{2+}-release messenger) and protein de-acetylation (O-acetylated ADP ribose), but the TRPM2 domain itself is an ineffective hydrolase. The channel is regulated by signaling pathways responsive to H2O2 and tumor-necrosis factor, suggesting that it may act as a sensor of intracellular oxidation/reduction, possibly during the oxidative burst of neutrophils.

TRPM3 forms a Ca^{2+}-permeant nonselective channel that is constitutively active when heterologously expressed. Its activity is increased by hypotonicity (200 mOsm per liter). TRPM3 is expressed primarily in kidney distal-collecting-duct epithelium and in the central nervous system.

TRPM4 and TRPM5 are the only monovalent-selective ion channels of the TRP family. They are widely distributed and may account for observed Ca^{2+}-activated 20–30 pS nonselective channel activities. They are activated through GPCRs coupled to PLC-dependent endoplasmic reticular Ca^{2+} release, perhaps by direct Ca^{2+} binding to the channel. However, relatively high $[Ca^{2+}]i$ is required to activate these channels, suggesting that they localize close to sites of Ca^{2+} release or that other modulators are important. Although their instantaneous I–V relationships are linear, the fraction of open channels increases at positive potentials. This voltage dependence is not mediated by divalent cation binding, suggesting an intrinsic voltage-sensing mechanism.

TRPM5 is found in cells expressing taste receptors. In an *in vivo* study in TrpM5-/- mice, it was shown that taste receptors T1R and T2R share a common signaling pathway involving PLC2 and TRPM5, to produce sweet, umami and bitter taste sensations. The authors concluded that InsP3, Ca^{2+} and thapsigargin-mediated store depletion did not activate TRPM5. However, it is possible that PIP2 or other molecules modulate its sensitivity to $[Ca^{2+}]i$.

TRPM6 and TRPM7 are unique among ion channels because they also contain functional kinase domains. TRPM7 passes little inward current under physiological conditions, is permeant to both Ca^{2+} and Mg^{2+}, and is inhibited by 0.6 mM intracellular free Mg^{2+}. In contrast to other GPCR-activated TRP channels, TRPM7 current increases slowly under whole-cell recording conditions and is inactivated by PIP2 hydrolysis by PLCbeta or PLCgamma. The function of the kinase domain is poorly understood and its substrates have not been identified. The kinase domain, in contrast to original reports, is not required for channel activation. The catalytic core of the kinase domain is similar to that of other eukaryotic protein kinases and to enzymes with ATP-grasp domains. The sensitivity of TRPM7 to physiological Mg–ATP levels has been suggested to have a central role in metabolic sensing or to serve as a mechanism to adjust cellular Mg^{2+} homeostasis. But a spontaneous human mutation in TRPM6 results in familial hypomagnesaemia with secondary hypocalcemia, suggesting that TRPM6 may be important for Mg^{2+} uptake in the kidney and intestine.

TRPM8 was identified as a messenger RNA that was upregulated in prostatic and other cancers. Its sensory role was recognized when it was isolated by expression cloning of a menthol receptor from trigeminal neurons. TRPM8 is a nonselective, outwardly rectifying channel that can be activated by cold (8–28 °C) and enhanced by 'cooling' compounds such as menthol and icilin. TRPM8 is widely expressed, but thought to function specifically as a thermosensor in TrkA+, small-diameter primary sensory neurons.

ANKTM1, a Ca^{2+}-permeant, nonselective channel homologous to Drosophila painless, is distinguished by 14 amino-terminal ankyrin repeats. It is activated by noxious cold temperature (<15 °C) but bears little similarity to menthol-sensitive TRPM8. It is found in a subset of nociceptive sensory dorsal root ganglion neurons, in the company of capsaicin-sensitive TRPV1, but not TRPM8. Interestingly, the Drosophila orthologue of ANKTM1 responds to warming (>27 °C) rather than to cooling when expressed in Xenopus oocytes. These observations are consistent with sensitivity to the surrounding membrane environment, but might also be

reconciled if the lowest energy state of the mammalian channel is the open configuration. Other TRP channels have not been systematically tested for temperature sensitivity, but such a comparison would clarify this issue.

The TRPP (polycystin) and TRPML (mucolipin) subfamilies

Polycystic kidney disease proteins PKD2, PKD2L1 and PKD2L2 are 6TM Ca^{2+}-permeant channels called TRPP2, TRPP3 and TRPP5, respectively. The much larger TRPP1, polycystin-REJ and polycystin-1L1 proteins are 11TM proteins that contain a C-terminal 6TM TRP-like channel domain. TRPP1 is not known to form a channel by itself, but it complexes with TRPP2 to form a Ca^{2+}-permeable nonselective cation channel70. Autosomal dominant polycystic kidney disease is caused by mutations in TRPP1 or TRPP2, leading to alterations in the polarization and function of cyst-lining epithelial cells. Trpp1-/- and Trpp2-/- mice die *in utero* with cardiac septal defects and cystic changes in nephrons and pancreatic ducts. The mouse ortholog of TRPP3 is deleted in krd mice, resulting in defects in the kidney and retina.

TRPP proteins have another role in development. Normal body asymmetry appears to arise from leftward extracellular flow generated by motor-protein-dependent rotation of monocilia on the ventral surface of the embryonic node. Motile monocilia generate nodal flow, and non-motile TRPP2-containing cilia sense nodal flow, initiating an asymmetric Ca^{2+} signal at the left nodal border. TRPP1 and TRPP2 both appear to be targeted to primary cilia cells of renal epithelia, where the channel complex is gated by fluid flow.

The mucolipins (MCOLN1, MCOLN2 and MCOLN3) are 6TM channels that are probably restricted to intracellular vesicles. Mutations in MCOLN1 (TRPML1) are associated with mucolipidosis type IV, a neurodegenerative lysosomal storage disorder. The defect appears to be in sorting or transport in the late endocytic pathway. Mutations in a Caenorhabditis elegans TRPML1 homologue, cup-5, cause excess lysosome formation and apoptosis in all cell types. TRPML3 is present in the cytoplasm of hair cells and the plasma membrane of stereocilia. TRPML3 is mutated in the varitint-waddler mouse, resulting in deafness and pigmentation defects.

TRP channel related disorders

Modulation of TRP channel protein levels, protein interactions, and function may be used to treat disorders including, but not intended to be limit to:

-Defects in osmoregulation, including brain edema, abdominal ascites, pulmonary effusion, pericardial effusion and bruising

-Defects in olfaction

-Defects in taste sensation

-Defects in hearing

-Peripheral pain syndromes, including painful neuromas, denervation pain, and pain due to averrent innervation

- Central pain syndromes, including thalamic pain, denervation pain, hyperpathia, and hyperalgesia

- Defect in thermoregulation and thermal sensation (nociception)

- Visual defects, including defects in phototransduction, glaucoma, retinal ischemia, idiopathic retinopathies, and macular degeneration

-Defects of vascular tone, including hypertension, orthostatic hypotension and defects of microvascular regulation including pulmonary, brain, myocardial edemas, and abdominal ascites

-Defects of airway resistance, including asthma

-Defects of myocardial function, including cardiomyopathies, myocardial ischemia, and cardiac arrhythmias

-Defects of bladder and ureteral function

-Defects of renal function.

-Defects of the immune system and inflammatory response

-Defects of tissue senescence/aging

-Defects of the endocrine system and metabolism, including familial hypomagnesemia with secondary hypocalcemia, diabetes and mucolipidosis type IV

-Cancer, including prostate [cancer] and melanoma

-Congenital malformations, including atrial septal defects, polycystic kidneys, congenital retinopathies and congenital deafness

-Defects in skin pigmentation

V. PDZ Protein and PL Protein Interactions

TABLES 6, 7, and 8 list PDZ proteins and other PL proteins which the current inventors have identified as binding to one another. This column provides the gene name for the PDZ portion of the GST-PDZ fusion that interacts with the PDZ-ligand to the left. Many of the genes listed in **TABLE 5** express more than one amino acid sequence, depending on alternative exon splicing and single amino acid changes. It is understood in the art that many alternatively spliced and point mutated forms of the same gene may exist in nature. As indicated supra, all peptides were biotinylated at the amino terminus and the amino acid sequences correspond to the C-terminal sequence of the gene name listed in column 1.

TABLE 5 lists the sequences of the PDZ domains cloned into a vector (PGEX-3X vector) for production of GST-PDZ fusion proteins (Pharmacia). More specifically, the first column (left to right) entitled "Gene Name" lists the name of the gene containing the PDZ domain. The second column labeled "GI or Acc#" is a unique Genbank identifier for the gene used to design primers for PCR amplification of the listed sequence. The next column labeled "Domain#" indicates the Pfam-predicted PDZ domain number, as numbered from the amino-terminus of the gene to the carboxy-terminus. The last column entitled "Sequence fused to GST Construct" (SEQ ID NO:s) provides the actual amino acid sequence inserted into the GST-PDZ expression vector as determined by DNA sequencing of the constructs.

TABLE 5: Sequences of PDZ Domains Cloned to Produce GST-PDZ Fusions

Gene Name	GI or Acc. #	Domain #	Sequence fused to GST Construct	SEQ ID NO:
AF6	430993	1	LRKEPEIITVTLKKQNGMGLSIVAAKGAGQDKLGIY VKSVVKGGAADVDGRLAAGDQLLSVDGRSLVGLS QERAAELMTRTSSVVTLEVAKQG	3
AIPC	12751451	1	LIRPSVISIIGLYKEKGKGLGFSIAGGRDCIRGQMGI FVKTIFPNGSAAEDGRLKEGDEILDVNGIPIKGLTF QEAIHTFKQIRSGLFVLTVRTKLVSPSLTNSS	4
AIPC	12751451	3	QSENEEDVCFIVLNRKEGSGLGFSVAGGTDVEPK SITVHRVFSQGAASQEGTMNRGDFLLSVNGASLA GLAHGNVLKVLHQAQLHKDALVVIKKGMDQPRPS NSS	5

AIPC	12751451	2	GISSLGRKTPGPKDRIVMEVTLNKEPRVGLGIGAC CLALENSPPGIYIHSLAPGSVAKMESNLSRGDQILE VNSVNVRHAALSKVHAILSKCPPGPVRLVIGRHPN PKVSEQEMDEVIARSTYQESKEANSS	6
AIPC	12751451	4	LGRSVAVHDALCVEVLKTSAGLGLSLDGGKSSVT GDGPLVIKRVYKGGAAEQAGIIEAGDEILAINGKPL VGLMHFDAWNIMKSVPEGPVQLLIRKHRNSS	7
ALP	2773059	1	REEGGMPQTVILPGPAPWGFRLSGGIDFNQPLVIT RITPGSKAAAANLCPGDVILAIDGFGTESMTHADA QDRIKAAAHQLCLKIDRGETHLWSPNSS	8
APXL1	13651263	1	ILVEVQLSGGAPWGFTLKGGREHGEPLVITKIEEG SKAAAVDKLLAGDEIVGINDIGLSGFRQEAICLVKG SHKTLKLVVKRNSS	9
CARD11	12382772	1	SVGHVRGPGPSVQHTTLNGDSLTSQLTLLGGNAR GSFVHSVKPGSLAEKAGLREGHQLLLLEGCIRGER QSVPLDTCTKEEAHWTIQRCSGPVTLHYKVNHEG YRK	10
CARD14	13129123	1	RRPARRILSQVTMLAFQGDALLEQISVIGGNLTGIFI HRVTPGSAADQMALRPGTQIVMVDYEASEPLFKA VLEDTTLEEAVGLLRRVDGFCCLSVKVNTDGYKR	11
CARD14	13129123	1	ILSQVTMLAFQGDALLEQISVIGGNLTGIFIHRVTPG SAADQMALRPGTQIVMVDYEASEPLFKAVLEDTTL EEAVGLLRRVDGFCCLSVKVNTDGYKRL	12
CASK	3087815	1	TRVRLVQFQKNTDEPMGITLKMNELNHCIVARIMH GGMIHRQGTLHVGDEIREINGISVANQTVEQLQKM LREMRGSITFKIVPSYRTQS	13
CNK1	3930780	1	LEQKAVLEQVQLDSPLGLEIHTTSNCQHFVSQVDT QVPTDSRLQIQPGDEVVQINEQVVVGWPRKNMVR ELLREPAGLSLVLKKIPIP	14
Cytohesin binding Protein	3192908	1	QRKLVTVEKQDNETFGFEIQSYRPQNQNACSSEM FTLICKIQEDSPAHCAGLQAGDVLANINGVSTEGFT YKQVVDLIRSSGNLLTIETLNG	15
Densin	16755892	1	RCLIQTKGQRSMDGYPEQFCVRIEKNPGLGFSISG GISGQGNPFKPSDKGIFVTRVQPDGPASNLLQPG DKILQANGHSFVHMEHEKAVLLLKSFQNTVDLVIQ RELTV	16
DLG 6 splice variant 2	AB053303	1	PTSPEIQELRQMLQAPHFKGATIKRHEMTGDILVA RIIHGGLAERSGLLYAGDKLVEVNGVSVEGLDPEQ VIHILAMSRGTIMFKVVPVSDPPVNSS	17
DLG 6, splice variant 1	14647140	1	PTSPEIQELRQMLQAPHFKALLSAHDTIAQKDFEPL LPPLPDNIPESEEAMRIVCLVKNQQPLGATIKRHEM TGDILVARIIHGGLAERSGLLYAGDKLVEVNGVSVE GLDPEQVIHILAMSRGTIMFKVVPVSDPPVNSS	18
DLG1	475816	1	IQVNGTDADYEYEEITLERGNSGLGFSIAGGTDNP HIGDDSSIFITKIITGGAAAQDGRLRVNDCILQVNEV DVRDVTHSKAVEALKEAGSIVRLYVKRRN	19
DLG1	475816	2	IQLIKGPKGLGFSIAGGVGNQHIPGDNSIYVTKIIEG GAAHKDGKLQIGDKLLAVNNVCLEEVTHEEAVTAL KNTSDFVYLKVAKPTSMYMNDGN	20
DLG1	475816	1,2	VNGTDADYEYEEITLERGNSGLGFSIAGGTDNPHI GDDSSIFITKIITGGAAAQDGRLRVNDCILQVNEVD VRDVTHSKAVEALKEAGSIVRLYVKRRKPVSEKIM EIKLIKGPKGLGFSIAGGVGNQHIPGDNSIYVTKIIE GGAAHKDGKLQIGDKLLAVNNVCLEEVTHEEAVTA LKNTSDFVYLKVAKPTSMYMNDGYA	21

DLG1	475816	3	ILHRGSTGLGFNIVGGEDGEGIFISFILAGGPADLSGELRKGDRIISVNSVDLRAASHEQAAAALKNAGQAVTIVAQYRPEEYSR	22
DLG2	12736552	3	IEGRGILEGEPRKVVLHKGSTGLGFNIVGGEDGEGIFVSFILAGGPADLSGELQRGDQILSVNGIDLRGASHEQAAAALKGAGQTVTIIAQHQPEDYARFEAKIHDLNSS	23
DLG2	12736552	1	ISYVNGTEIEYEFEEITLERGNSGLGFSIAGGTDNPHIGDDPGIFITKIIPGGAAAEDGRLRVNDCILRVNEVDVSEVSHSKAVEALKEAGSIVRLYVRRR	24
DLG2	12736552	2	IPILETVVEIKLFKGPKGLGFSIAGGVGNQHIPGDNSIYVTKIIDGGAAQKDGRLQVGDRLLMVNNYSLEEVTHEEAVAILKNTSEVVYLKVGKPTTIYMTDPYGPPNSSLTD	25
DLG5	3650451	1	GIPYVEEPRHVKVQKGSEPLGISIVSGEKGGIYVSKVTVGSIAHQAGLEYGDQLLEFNGINLRSATEQQARLIIGQQCDTITILAQYNPHVHQLRNSSLTD	26
DLG5	3650451	2	GILAGDANKKTLEPRVVFIKKSQLELGVHLCGGNLHGVFVAEVEDDSPAKGPDGLVPGDLILEYGSLDVRNKTVEEVYVEMLKPRDGVRLKVQYRPEEFIVTD	27
DVL1	2291005	1	LNIVTVTLNMERHHFLGISIVGQSNDRGDGGIYIGSIMKGGAVAADGRIEPGDMLLQVNDVNFENMSNDDAVRVLREIVSQTGPISLTVAKCW	28
DVL2	2291007	1	LNIITVTLNMEKYNFLGISIVGQSNERGDGGIYIGSIMKGGAVAADGRIEPGDMLLQVNDMNFENMSNDDAVRVLRDIVHKPGPIVLTVAKCWDPSPQNS	29
DVL3	6806886	1	IITVTLNMEKYNFLGISIVGQSNERGDGGIYIGSIMKGGAVAADGRIEPGDMLLQVNEINFENMSNDDAVRVLREIVHKPGPITLTVAKCWDPSP	30
EBP50	3220018	2	QQRELRPRLCTMKKGPSGYGFNLHSDKSKPGQFIRSVDPDSPAEASGLRAQDRIVEVNGVCMEGKQHGDVVSAIRAGGDETKLLVVDRETDEFFKNSS	31
EBP50	3220018	1	GIQMSADAAAGAPLPRLCCLEKGPNGYGFHLHGEKGKLGQYIRLVEPGSPAEKAGLLAGDRLVEVNGENVEKETHQQVVSRIRAALNAVRLLVVDPETDEQLQKLGVQVREELLRAQEAPGQAEPPAAAEVQGAGNENEPREADKSHPEQRELRN	32
EBP50	3220018	1,2	GIQMSADAAAGAPLPRLCCLEKGPNGYGFHLHGEKGKLGQYIRLVEPGSPAEKAGLLAGDRLVEVNGENVEKETHQQVVSRIRAALNAVRLLVVDPETDEQLQKLGVQVREELLRAQEAPGQAEPPAAAEVQGAGNENEPREADKSHPEQRELRPRLCTMKKGPSGYGFNLHSDKSKPGQFIRSVDPDSPAEASGLRAQDRIVEVNGVCMEGKQHGDVVSAIRAGGDETKLLVVDRETDEFFK	33
EBP50	3220018	1	QMSADAAAGAPLPRLCCLEKGPNGYGFHLHGEKGKLGQYIRLVEPGSPAEKAGLLAGDRLVEVNGENVEKETHQQVVSRIRAALNAVRLLVVDPETDEQLQKLGVQVREELLRAQEAPGQAEPPAAAEVQGAGNENEPREADKSHPEQRELRNSS	34
ELFIN 1	2957144	1	LTTQQIDLQGPGPWGFRLVGGKDFEQPLAISRVTPGSKAALANLCIGDVITAIDGENTSNMTHLEAQNRIKGCTDNLTLTVARSEHKVWSPLVTNSSW	35

ENIGMA	561636	1	IFMDSFKVVLEGPAPWGFRLQGGKDFNVPLSISRL TPGGKAAQAGVAVGDWVLSIDGENAGSLTHIEAQ NKIRACGERLSLGLSRAQPV	36
ERBIN	8923908	1	QGHELAKQEIRVRVEKDPELGFSISGGVGGRGNP FRPDDDGIFVTRVQPEGPASKLLQPGDKIIQANGY SFINIEHGQAVSLLKTFQNTVELIIVREVSS	37
FLJ00011	10440352	1	KNPSGELKTVTLSKMKQSLGISISGGIESKVQPMVK IEKIFPGGAAFLSGALQAGFELVAVDGENLEQVTH QRAVDTIRRAYRNKAREPMELVVRVPGPSPRPSP SD	38
FLJ11215	11436365	1	EGHSHPRVVELPKTEEGLGFNIMGGKEQNSPIYIS RIIPGGIADRHGGLKRGDQLLSVNGVSVEGEHHEK AVELLKAAQGKVKLVVRYTPKVLEEME	39
FLJ12428	BC012040	1	PGAPYARKTFTIVGDAVGWGFVVRGSKPCHIQAV DPSGPAAAAGMKVCQFVVSVNGLNVLHVDYRTVS NLILTGPRTIVMEVMEELEC	40
FLJ12615	10434209	1	GQYGGETVKIVRIEKARDIPLGATVRNEMDSVIISRI VKGGAAEKSGLLHEGDEVLEINGIEIRGKDVNEVF DLLSDMHGTLTFVLIPSQQIKPPPA	41
FLJ21687	10437836	1	KPSQASGHFSVELVRGYAGFGLTLGGGRDVAGDT PLAVRGLLKDGPAQRCGRLEVGDLVLHINGESTQ GLTHAQAVERIRAGGPQLHLVIRRPLETHPGKPRG V	42
FLJ31349	AK055911	1	PVMSQCACLEEVHLPNIKPGEGLGMYIKSTYDGLH VITGTTENSPADRSQKIHAGDEVTQVNQQTVVGW QLKNLVKKLRENPTGVVLLLKKRPTGSFNFTP	43
FLJ32798	AK057360	1	IDDEEDSVKIIRLVKNREPLGATIKKDEQTGAIIVARI MRGGAADRSGLIHVGDELREVNGIPVEDKRPEEII QILAQSQGAITFKIIPGSKEETPS	44
GORASP 2	13994253	1,2	MGSSQSVEIPGGGTEGYHVLRVQENSPGHRAGLE PFFDFIVSINGSRLNKDNDTLKDLLKANVEKPVKML IYSSKTLELRETSVTPSNLWGGQGLLGVSIRFCSF DGANENVWHVLEVESNSPAALAGLRPHSDYIIGAD TVMNESEDLFSLIETHEAKPLKLYVYNTDTDNCRE VIITPNSAWGGEGSLGCGIGYGYLHRIPTRPFEEG KKISLPGQMAGTPITPLKDGFTEVQLSSVNPPSLSP PGTTGIEQSLTGLSISSTPPAVSSVLSTGVPTVPLL PPQVNQSLTSVPPMNPATTLPGLMPLPAGLPNLP NLNLNLPAPHIMPGVGLPELVNPGLPPLPSMPPRN LPGIAPLPLPSEFLPSFPLVPESSSAASSGELLSSL PPTSNAPSDPATTTAKADAASSLTVDVTPPTAKAP TTVEDRVGDSTPVSEKPVSAAVDANASESP	45
GORASP 2	13994253	2	NENVWHVLEVESNSPAALAGLRPHSDYIIGADTVM NESEDLFSLIETHEAKPLKLYVYNTDTDNCREVIITP NSAWGGEGSLGCGIGYGYLHRIPTR	46
GORASP 2	13994253	1	MGSSQSVEIPGGGTEGYHVLRVQENSPGHRAGLE PFFDFIVSINGSRLNKDNDTLKDLLKANVEKPVKML IYSSKTLELRETSVTPSNLWGGQGLLGVSIRFCSF DGANE	47
GORASP 1	29826292	2	RASEQVWHVLDVEPSSPAALAGLRPYTDYVVGSD QILQESEDFFTLIESHEGKPLKLMVYNSKSDSCREV TVTPNAAWGGEGSLGCGIGYGYLHRIPTQ	48
GORASP 1	29826292	1	MGLGVSAEQPAGGAEGFHLHGVQENSPAQQAGL EPYFDFIITIGHSRLNKENDTLKALLKANVEKPVKLE VFNMKTMRVREVEVVPSNMWGGQGLLGASVRFC	49

				SFRRASE
GORASP 1	29826292	1,2	MGLGVSAEQPAGGAEGFHLHGVQENSPAQQAGL EPYFDFIITIGHSRLNKENDTLKALLKANVEKPVKLE VFNMKTMRVREVEVVPSNMWGGQGLLGASVRFC SFRRASEQVWHVLDVEPSSPAALAGLRPYTDYVV GSDQILQESEDFFTLIESHEGKPLKLMVYNSKSDS CREVTVTPNAAWGGEGSLGCGIGYGYLHRIPTQP PSYHKKPPGTPPPSALPLGAPPPDALPPGPTPEDS PSLETGSRQSDYMEALLQAPGSSMEDPLPGPGSP SHSAPDPDGLPHFMETPLQPPPPVQRVMDPGFLD VSGISLLDNSNASVWPSLPSSTELTTTAVSTSGPE DICSSSSSHERGGEATWSGSEFEVSFLDSPGAQA QADHLPQLTLPDSLTSAASPEDGLSAELLEAQAEE EPASTEGLDTGTEAEGLDSQAQISTTE	50
GRIP 1	4539083	6	IYTVELKRYGGPLGITISGTEEPFDPIIISSLTKGGLA ERTGAIHIGDRILAINSSSLKGKPLSEAIHLLQMAGE TVTLKIKKQTDAQSA	51
GRIP 1	4539083	1	VVELMKKEGTTLGLTVSGGIDKDGKPRVSNLRQG GIAARSDQLDVGDYIKAVNGINLAKFRHDEIISLLKN VGERVVLEVEYE	52
GRIP 1	4539083	3	HVATASGPLLVEVAKTPGASLGVALTTSMCCNKQ VIVIDKIKSASIADRCGALHVGDHILSIDGTSMEYCT LAEATQFLANTTDQVKLEILPHHQTRLALKGPNSS	53
GRIP 1	4539083	7	IMSPTPVELHKVTLYKDSDMEDFGFSVADGLLEKG VYVKNIRPAGPGDLGGLKPYDRLLQVNHVRTRDF DCCLVVPLIAESGNKLDLVISRNPLA	54
GRIP 1	4539083	4	IYTVELKRYGGPLGITISGTEEPFDPIIISSLTKGGLA ERTGAIHIGDRILAINSSSLKGKPLSEAIHLLQMAGE TVTLKIKKQTDAQSA	55
GRIP 1	4539083	5	IMSPTPVELHKVTLYKDSDMEDFGFSVADGLLEKG VYVKNIRPAGPGDLGGLKPYDRLLQVNHVRTRDF DCCLVVPLIAESGNKLDLVISRNPLA	56
GTPase activating enzyme	2389008	1	SRGCETRELALPRDGQGRLGFEVDAEGFVTHVER FTFAETAGLRPGARLLRVCGQTLPSLRPEAAAQLL RSAPKVCVTVLPPDESGRP	57
Guanine exchange factor	6650765	1	CSVMIFEVVEQAGAIILEDGQELDSWYVILNGTVEI SHPDGKVENLFMGNSFGITPTLDKQYMHGIVRTKV DDCQFVCIAQQDYWRILNHVEKNTHKVEEEGEIVM VH	58
HEMBA 1000505	10436367	2	PRETVKIPDSADGLGFQIRGFGPSVVHAVGRGTVA AAAGLHPGQCIIKVNGINVSKETHASVIAHVTACRK YRRPTKQDSIQ	59
HEMBA 1000505	10436367	1	LENVIAKSLLIKSNEGSYGFGLEDKNKVPIIKLVEKG SNAEMAGMEVGKKIFAINGDLVFMRPFNEVDCFLK SCLNSRKPLRVLVSTKP	60
HEMBA 1003117	7022001	1	EDFCYVFTVELERGPSGLGMGLIDGMHTHLGAPG LYIQTLLPGSPAAADGRLSLGDRILEVNGSSLLGLG YLRAVDLIRHGGKKMRFLVAKSDVETAKKI	61
hShroom	18652858	1	IYLEAFLEGGAPWGFTLKGGLEHGEPLIISKVEEGG KADTLSSKLQAGDEVVHINEVTLSSSRKEAVSLVK GSYKTLRLVVRRDVCTDPGH	62
HSPC227	7106843	1	NNELTQFLPRTITLKKPPGAQLGFNIRGGKASQLGI FISKVIPDSDAHRAGLQEGDQVLAVNDVDFQDIEH	63

SKAVEILKTAREISMRVRFFPYNYHRQKE

HTRA 3	AY040094	1	FLTEFQDKQIKDWKKRFIGIRMRTITPSLVDELKAS NPDFPEVSSGIYVQEVAPNSPSQRGGIQDGDIIVK VNGRPLVDSSELQEAVLTESPLLLEVRRGNDDLLF S	64
HTRA 4	AL576444	1	NKKYLGLQMLSLTVPLSEELKMHYPDFPDVSSGVY VCKVVEGTAAQSSGLRDHDVIVNINGKPITTTTDVV KALDSDSLSMAVLRGKDNLLLTV	65
INADL	2370148	3	PGSDSSLFETYNVELVRKDGQSLGIRIVGYVGTSH TGEASGIYVKSIIPGSAAYHNGHIQVNDKIVAVDGV NIQGFANHDVVEVLRNAGQVVHLTLVRRKTSSSTS RIHRD	66
INADL	2370148	8	PATCPIVPGQEMIIEISKGRSGLGLSIVGGKDTPLNA IVIHEVYEEGAAARDGRLWAGDQILEVNGVDLRNS SHEEAITALRQTPQKVRLVVY	67
INADL	2370148	2	LPETVCWGHVEEVELINDGSGLGFGIVGGKTSGVV VRTIVPGGLADRDGRLQTGDHILKIGGTNVQGMTS EQVAQVLRNCGNSVRMLVARDPAGDIQSPI	68
INADL	2370148	6	PNFSHWGPPRIVEIFREPNVSLGISIVVGQTVIKRLK NGEELKGIFIKQVLEDSPAGKTNALKTGDKILEVSG VDLQNASHSEAVEAIKNAGNPVVFIVQSLSSTPRVI PNVHNKANSS	69
INADL	2370148	7	PGELHIIELEKDKNGLGLSLAGNKDRSRMSIFVVGI NPEGPAAADGRMRIGDELLEINNQILYGRSHQNAS AIIKTAPSKVKLVFIRNEDAVNQMANSS	70
INADL	2370148	5	LSSPEVKIVELVKDCKGLGFSILDYQDPLDPTRSVI VIRSLVADGVAERSGGLLPGDRLVSVNEYCLDNTS LAEAVEILKAVPPGLVHLGICKPLVEFIVTD	71
INADL	2370148	1	IWQIEYIDIERPSTGGLGFSVVALRSQNLGKVDIFVK DVQPGSVADRDQRLKENDQILAINHTPLDQNISHQ QAIALLQQTTGSLRLIVAREPVHTKSSTSSSE	72
INADL	2370148	4	NSDDAELQKYSKLLPIHTLRLGVEVDSFDGHHYISS IVSGGPVDTLGLLQPEDELLEVNGMQLYGKSRREA VSFLKEVPPPFTLVCCRRLFDDEAS	73
KIAA0313	7657260	1	HLRLLNIACAAKAKRRLMTLTKPSREAPLPFILLGG SEKGFGIFVDSVDSGSKATEAGLKRGDQILEVNGQ NFENIQLSKAMEILRNNTHLSITVKTNLFVFKELLTR LSEEKRNGAP	74
KIAA0316	6683123	1	IPPAPRKVEMRRDPVLGFGFVAGSEKPVVVRSVTP GGPSEGKLIPGDQIVMINDEPVSAAPRERVIDLVRS CKESILLTVIQPYPSPK	75
KIAA0340	2224620	1	LNKRTTMPKDSGALLGLKVVGGKMTDLGRLGAFIT KVKKGSLADVVGHLRAGDEVLEWNGKPLPGATNE EVYNIILESKSEPQVEIIVSRPIGDIPRIHRD	76
KIAA0380	2224700	1	RCVIIQKDQHGFGFTVSGDRIVLVQSVRPGGAAMK AGVKEGDRIIKVNGTMVTNSSHLEVVKLIKSGAYVA LTLLGS	77
KIAA0382	7662087	1	ILVQRCVIIQKDDNGFGLTVSGDNPVFVQSVKEDG AAMRAGVQTGDRIIKVNGTLVTHSNHLEVVKLIKSG SYVALTVQGRPPGNSS	78
KIAA0440	2662160	1	SVEMTLRRNGLGQLGFHVNYEGIVADVEPYGYAW QAGLRQGSRLVEICKVAVATLSHEQMIDLLRTSVT VKVVIIPPH	79

KIAA0545	14762850	1	LKVMTSGWETVDMTLRRNGLGQLGFHVKYDGTV AEVEDYGFAWQAGLRQGSRLVEICKVAVVTLTHD QMIDLLRTSVTVKVVIIPPFEDGTPRRGW	80
KIAA0559	3043641	1	HYIFPHARIKITRDSKDHTVSGNGLGIRIVGGKEIPG HSGEIGAYIAKILPGGSAEQTGKLMEGMQVLEWN GIPLTSKTYEEVQSIISQQSGEAEICVRLDLNML	81
KIAA0613	3327039	1	SYSVTLTGPGPWGFRLQGGKDFNMPLTISRITPGS KAAQSQLSQGDLVVAIDGVNTDTMTHLEAQNKIKS ASYNLSLTLQKSKNSS	82
KIAA0858	4240204	1	FSDMRISINQTPGKSLDFGFTIKWDIPGIFVASVEA GSPAEFSQLQVDDEIIAINNTKFSYNDSKEWEEAM AKAQETGHLVMDVRRYGKAGSPE	83
KIAA0902	4240292	1	QSAHLEVIQLANIKPSEGLGMYIKSTYDGLHVITGT TENSPADRCKKIHAGDEVIQVNHQTVVGWQLKNL VNALREDPSGVILTLKKRPQSMLTSAPA	84
KIAA0967	4589577	1	ILTQTLIPVRHTVKIDKDTLLQDYGFHISESLPLTVVA VTAGGSAHGKLFPGDQILQMNNEPAEDLSWERAV DILREAEDSLSITVVRCTSGVPKSSNSS	85
KIAA1202	6330421	1	RSFQYVPVQLQGGAPWGFTLKGGLEHCEPLTVSK IEDGGKAALSQKMRTGDELVNINGTPLYGSRQEAL ILIKGSFRILKLIVRRRNAPVS	86
KIAA1222	6330610	1	ILEKLELFPVELEKDEDGLGISIIGMGVGADAGLEKL GIFVKTVTEGGAAQRDGRIQVNDQIVEVDGISLVG VTQNFAATVLRNTKGNVRFVIGREKPGQVSE	87
KIAA1284	6331369	1	KDVNVYVNPKKLTVIKAKEQLKLLEVLVGIIHQTKW SWRRTGKQGDGERLVVHGLLPGGSAMKSGQVLI GDVLVAVNDVDVTTENIERVLSCIPGPMQVKLTFE NAYDVKRET	88
KIAA1389	7243158	1	TRGCETVEMTLRRNGLGQLGFHVNFEGIVADVEP FGFAWKAGLRQGSRLVEICKVAVATLTHEQMIDLL RTSVTVKVVIIQPHDDGSPRR	89
KIAA1415	7243210	1	VENILAKRLLILPQEEDYGFDIEEKNKAVVVKSVQR GSLAEVAGLQVGRKIYSINEDLVFLRPFSEVESILN QSFCSRRPLRLLVATKAKEIIKIP	90
KIAA1526	5817166	1	PDSAGPGEVRLVSLRRAKAHEGLGFSIRGGSEHG VGIYVSLVEPGSLAEKEGLRVGDQILRVNDKSLAR VTHAEAVKALKGSKKLVLSVYSAGRIPGGYVTNH	91
KIAA1526	5817166	2	LQGGDEKKVNLVLGDGRSLGLTIRGGAEYGLGIYIT GVDPGSEAEGSGLKVGDQILEVNGRSFLNILHDEA VRLLKSSRHLILTVKDVGRLPHARTTVDE	92
KIAA1620	10047316	1	LRRAELVEIIVETEAQTGVSGINVAGGGKEGIFVRE LREDSPAARSLSLQEGDQLLSARVFFENFKYEDAL RLLQCAEPYKVSFCLKRTVPTGDLALR	93
KIAA1719	1267982	5	IQTTGAVSYTVELKRYGGPLGITISGTEEPFDPIVIS GLTKRGLAERTGAIHVGDRILAINNVSLKGRPLSEAI HLLQVAGETVTLKIKKQLDR	94
KIAA1719	1267982	6	ILEMEELLLPTPLEMHKVTLHKDPMRHDFGFSVSD GLLEKGVYVHTVRPDGPAHRGGLQPFDRVLQVNH VRTRDFDCCLAVPLLAEAGDVLELIISRKPHTAHSS	95
KIAA1719	1267982	2	IHTVANASGPLMVEIVKTPGSALGISLTTTSLRNKS VITIDRIKPASVVDRSGALHPGDHILSIDGTSMEHCS LLEATKLLASISEKVRLEILPVPQSQRPL	96
KIAA1719	1267982	1	ITVVELIKKEGSTLGLTISGGTDKDGKPRVSNLRPG GLAARSDLLNIGDYIRSVNGIHLTRLRHDEIITLLKN VGERVVLEVEY	97

KIAA1719	1267982	3	IQIVHTETTEVVLCGDPLSGFGLQLQGGIFATETLS SPPLVCFIEPDSPAERCGLLQVGDRVLSINGIATED GTMEEANQLLRDAALAHKVVLEVEFDVAESV	98
KIAA1719	1267982	1	ILDVSLYKEGNSFGFVLRGGAHEDGHKSRPLVLTY VRPGGPADREGSLKVGDRLLSVDGIPLHGASHAT ALATLRQCSHEALFQVEYDVATP	99
KIAA1719	1267982	4	QFDVAESVIPSSGTFHVKLPKKRSVELGITISSASR KRGEPLIISDIKKGSVAHRTGTLEPGDKLLAIDNIRL DNCPMEDAVQILRQCEDLVKLKIRKDEDN	100
LIM mystique	12734250	1	MALTVDVAGPAPWGFRITGGRDFHTPIMVTKVAE RGKAKDADLRPGDIIVAINGESAEGMLHAEAQSKIR QSPSPLRLQLDRSQATSPGQT	101
LIM protein	3108092	1	SNYSVSLVGPAPWGFRLQGGKDFNMPLTISSLKD GGKAAQANVRIGDVVLSIDGINAQGMTHLEAQNKI KGCTGSLNMTLQRAS	102
LIMK1	4587498	1	TLVEHSKLYCGHCYYQTVVTPVIEQILPDSPGSHLP HTVTLVSIPASSHGKRGLSVSIDPPHGPPGCGTEH SHTVRVQGVDPGCMSPDVKNSIHVGDRILEINGTP IRNVPLDEIDLLIQETSRLLQLTLEHD	103
LIMK2	1805593	1	PYSVTLISMPATTEGRRGFSVSVESACSNYATTVQ VKEVNRMHISPNNRNAIHPGDRILEINGTPVRTLRV EEVEDAISQTSQTLQLLIEHD	104
LIM-RIL	1085021	1	IHSVTLRGPSPWGFRLVGRDFSAPLTISRVHAGSK ASLAALCPGDLIQAINGESTELMTHLEAQNRIKGCH DHLTLSVSRPE	105
LU-1	U52111	1	VCYRTDDEEDLGIYVGEVNPNSIAAKDGRIREGDRI IQINGVDVQNREEAVAILSQEENTNISLLVARPESQ LA	106
MAGI 1	3370997	2	IPATQPELITVHIVKGPMGFGFTIADSPGGGGQRVK QIVDSPRCRGLKEGDLIVEVNKKNVQALTHNQVVD MLVECPKGSEVTLLVQRGGNSS	107
MAGI 1	3370997	5	IPDYQEQDIFLWRKETGFGFRILGGNEPGEPIYIGHI VPLGAADTDGRLRSGDELICVDGTPVIGKSHQLVV QLMQQAAKQGHVNLTVRRKVVFAVPKTENSS	108
MAGI 1	3370997	4	IPGVVSTVVQPYDVEIRRGENEGFGFVIVSSVSRP EAGTTFAGNACVAMPHKIGRIIEGSPADRCGKLKV GDRILAVNGCSITNKSHSDIVNLIKEAGNTVTLRIIP GDESSNAEFIVTD	109
MAGI 1	3370997	1	IPSELKGKFIHTKLRKSSRGFGFTVVGGDEPDEFL QIKSLVLDGPAALDGKMETGDVIVSVNDTCVLGHT HAQVVKIFQSIPIGASVDLELCRGYPLPFDPDGIHRD	110
MAGI 1	3370997	3	QATQEQDFYTVELERGAKGFGFSLRGGREYNMDL YVLRLAEDGPAERCGKMRIGDEILEINGETTKNMK HSRAIELIKNGGRRVRLFLKRG	111
Magi 2	2947231	1	REKPLFTRDASQLKGTFLSTTLKKSNMGFGFTIIGG DEPDEFLQVKSVIPDGPAAQDGKMETGDVIVYINE VCVLGHTHADVVKLFQSVPIGQSVNLVLCRGYP	112
Magi 2	2947231	3	HYKELDVHLRRMESGFGFRILGGDEPGQPILIGAVI AMGSADRDGRLHPGDELVYVDGIPVAGKTHRYVI DLMHHAARNGQVNLTVRRKVLCG	113
Magi 2	2947231	4	EGRGISSHSLQTSDAVIHRKENEGFGFVIISSLNRP ESGSTITVPHKIGRIIDGSPADRCAKLKVGDRILAVN GQSIINMPHADIVKLIKDAGLSVTLRIIPQEEL	114

Magi 2	2947231	2	LSGATQAELMTLTIVKGAQGFGFTIADSPTGQRVK QILDIQGCPGLCEGDLIVEINQQNVQNLSHTEVVDI LKDCPIGSETSLIIHRGGFF	115
Magi 2	2947231	5	LSDYRQPQDFDYFTVDMEKGAKGFGFSIRGGREY KMDLYVLRLAEDGPAIRNGRMRVGDQIIEINGEST RDMTHARAIELIKSGGRRVRLLLKRGTGQ	116
Magi 2	2947231	6	HESVIGRNPEGQLGFELKGGAENGQFPYLGEVKP GKVAYESGSKLVSEELLLEVNETPVAGLTIRDVLAV IKHCKDPLRLKCVKQGGIHR	117
MAGI 3	10047344?	2	ASSGSSQPELVTIPLIKGPKGFGFAIADSPTGQKVK MILDSQWCQGLQKGDIIKEIYHQNVQNLTHLQVVE VLKQFPVGADVPLLILRGGPPSPTKTAKM	118
MAGI 3	10047344	5	QNLGCYPVELERGPRGFGFSLRGGKEYNMGLFIL RLAEDGPAIKDGRIHVGDQIVEINGEPTQGITHTRAI ELIQAGGNKVLLLLRPGTGLIPDHGLA	119
MAGI 3	10047344	3	LYEDKPPNTKDLDVFLRKQESGFGFRVLGGDGPD QSIYIGAIIPLGAAEKDGRLRAADELMCIDGIPVKGK SHKQVLDLMTTAARNGHVLLTVRRKIFYGEKQPED DS	120
MAGI 3	10047344	1	PSQLKGVLVRASLKKSTMGFGFTIIGGDRPDEFLQ VKNVLKDGPAAQDGKIAPGDVIVDINGNCVLGHTH ADVVQMFQLVPVNQYVNLTLCRGYPLPDDSED	121
MAGI 3	10047344	4	PAPQEPYDVVLQRKENEGFGFVILTSKNKPPPGVI PHKIGRVIEGSPADRCGKLKVGDHISAVNGQSIVEL SHDNIVQLIKDAGVTVTLTVIAEEEHHGPPS	122
MAST1	4589589	1	GLRSPITIQRSGKKYGFTLRAIRVYMGDTDVYSVH HIVWHVEEGGPAQEAGLCAGDLITHVNGEPVHGM VHPEVVELILKSGNKVAVTTTPFEN	123
MAST2	3882334	1	ISALGSMRPPIIIHRAGKKYGFTLRAIRVYMGDSDV YTVHHMVWHVEDGGPASEAGLRQGDLITHVNGE PVHGLVHTEVVELILKSGNKVAISTTPLENSS	124
MAST3	3043645	1	LCGSLRPPIVIHSSGKKYGFSLRAIRVYMGDSDVYT VHHVVWSVEDGSPAQEAGLRAGDLITHINGESVL GLVHMDVVELLLKSGNKISLRTTALENTSIKVG	125
MAST4	2224546	1	PHQPIVIHSSGKNYGFTIRAIRVYVGDSDIYTVHHIV WNVEEGSPACQAGLKAGDLITHINGEPVHGLVHTE VIELLLKSGNKVSITTTPF	126
MGC5395	BC012477	1	PAKMEKEETTRELLLPNWQGSGSHGLTIAQRDDG VFVQEVTQNSPAARTGVVKEGDQIVGATIYFDNLQ SGEVTQLLNTMGHHTVGLKLHRKGDRSPNSS	127
MINT1	2625024	1	SENCKdVFIEKQKGEILGVVIVESGWGSILPTVIIAN MMHGGPAEKSGKLNIGDQIMSINGTSLVGLPLSTC QSIIKGLKNQSRVKLNIVRCPPVNSS	128
MINT1	2625024	1,2	SENCKDVFIEKQKGEILGVVIVESGWGSILPTVIIAN MMHGGPAEKSGKLNIGDQIMSINGTSLVGLPLSTC QSIIKGLENQSRVKLNIVRCPPVTTVLIRRPDLRYQL GFSVQNGIICSLMRGGIAERGGVRVGHRIIEINGQS VVATPHEKIVHILSNAVGEIHMKTMPAAMYRLL	129
MINT1	2625024	2	LRCPPVTTVLIRRPDLRYQLGFSVQNGIICSLMRGG IAERGGVRVGHRIIEINGQSVVATPHEKIVHILSNAV GEIHMKTMPAAMYRLLNSS	130
MINT3	3169808	1	HNGDLDHFSNSDNCREVHLEKRRGEGLGVALVES GWGSLLPTAVIANLLHGGPAERSGALSIGDRLTAIN GTSLVGLPLAACQAAVRETKSQTSVTLSIVHCPPV T	131

MINT3	3169808	2	PVTTAIIHRPHAREQLGFCVEDGIICSLLRGGIAERG GIRVGHRIIEINGQSVVATPHARIIELLTEAYGEVHIK TMPAATYRLLTGNSS	132
MINT3	3169808	1	LSNSDNCREVHLEKRRGEGLGVALVESGWGSLLP TAVIANLLHGGPAERSGALSIGDRLTAINGTSLVGL PLAACQAAVRETKSQTSVTLSIVHCPPVTTAIM	133
MPP1	189785	1	RKVRLIQFEKVTEEPMGITLKLNEKQSCTVARILHG GMIHRQGSLHVGDEILEINGTNVTNHSVDQLQKAM KETKGMISLKVIPNQ	134
MPP2	939884	1	PVPPDAVRMVGIRKTAGEHLGVTFRVEGGELVIAR ILHGGMVAQQGLLHVGDIIKEVNGQPVGSDPRALQ ELLRNASGSVILKILPNYQ	135
MPP3	21536463	1	NIDEDFDEESVKIVRLVKNKEPLGATIRRDEHSGAV VVARIMRGGAADRSGLVHVGDELREVNGIAVLHK RPDEISQILAQSQGSITLKIIPATQEEDR	136
MUPP1	2104784	5	WEAGIQHIELEKGSKGLGFSILDYQDPIDPASTVIIIR SLVPGGIAEKDGRLLPGDRLMFVNDVNLENSSLEE AVEALKGAPSGTVRIGVAKPLPLSPEE	137
MUPP1	2104784	12	LQGLRTVEMKKGPTDSLGISIAGGVGSPLGDVPIFI AMMHPTGVAAQTQKLRVGDRIVTICGTSTEGMTH TQAVNLLKNASGSIEMQVVAGGDVSV	138
MUPP1	2104784	2	PVHWQHMETIELVNDGSGLGFGIIGGKATGVIVKTI LPGGVADQHGRLCSGDHILKIGDTDLAGMSSEQV AQVLRQCGNRVKLMIARGAIEERTAPT	139
MUPP1	2104784	3	QESETFDVELTKNVQGLGITIAGYIGDKKLEPSGIFV KSITKSSAVEHDGRIQIGDQIIAVDGTNLQGFTNQQ AVEVLRHTGQTVLLTLMRRGMKQEA	140
MUPP1	2104784	11	KEEEVCDTLTIELQKKPGKGLGLSIVGKRNDTGVF VSDIVKGGIADADGRLMQGDQILMVNGEDVRNAT QEAVAALLKCSLGTVTLEVGRIKAGPFHS	141
MUPP1	2104784	8	LTGELHMIELEKGHSGLGLSLAGNKDRSRMSVFIV GIDPNGAAGKDGRLQIADELLEINGQILYGRSHQN ASSIIKCAPSKVKIIFIRNKDAVNQ	142
MUPP1	2104784	13	LGPPQCKSITLERGPDGLGFSIVGGYGSPHGDLPI YVKTVFAKGAASEDGRLKRGDQIIAVNGQSLEGVT HEEAVAILKRTKGTVTLMVLS	143
MUPP1	2104784	6	RNVSKESFERTINIAKGNSSLGMTVSANKDGLGMI VRSIIHGGAISRDGRIAIGDCILSINEESTISVTNAQA RAMLRRHSLIGPDIKITYVPAEHLEE	144
MUPP1	2104784	10	LPGCETTIEISKGRTGLGLSIVGGSDTLLGAIIIHEVY EEGAACKDGRLWAGDQILEVNGIDLRKATHDEAIN VLRQTPQRVRLTLYRDEAPYKE	145
MUPP1	2104784	7	LNWNQPRRVELWREPSKSLGISIVGGRGMGSRLS NGEVMRGIFIKHVLEDSPAGKNGTLKPGDRIVEVD GMDLRDASHEQAVEAIRKAGNPVVFMVQSIINRPR KSPLPSLL	146
MUPP1	2104784	9	LSSFKNVQHLELPKDQGGLGIAISEEDTLSGVIIKSL TEHGVAATDGRLKVGDQILAVDDEIVVGYPIEKFIS LLKTAKMTVKLTIHAENPDSQ	147
MUPP1	2104784	1	QGRHVEVFELLKPPSGGLGFSVVGLRSENRGELGI FVQEIQEGSVAHRDGRLKETDQILAINGQALDQTIT HQQAISILQKAKDTVQLVIARGSLPQLV	148
MUPP1	2104784	4	LNYEIVVAHVSKFSENSGLGISLEATVGHHFIRSVL PEGPVGHSGKLFSGDELLEVNGITLLGENHQDVVN ILKELPIEVTMVCCRRTVPPT	149

NeDLG	10863920	2	ITLLKGPKGLGFSIAGGIGNQHIPGDNSIYITKIIEGG AAQKDGRLQIGDRLLAVNNTNLQDVRHEEAVASLK NTSDMVYLKVAKPGSLE	150
NeDLG	10863920	1	IQYEEIVLERGNSGLGFSIAGGIDNPHVPDDPGIFIT KIIPGGAAAMDGRLGVNDCVLRVNEVEVSEVVHSR AVEALKEAGPVVRLVVRRRQN	151
NeDLG	10863920	3	ILLHKGSTGLGFNIVGGEDGEGIFVSFILAGGPADL SGELRRGDRILSVNGVNLRNATHEQAAAALKRAG QSVTIVAQYRPEEYSRFESKIHDLREQMMNSSMS SGSGSLRTSEKRSLE	152
NeDLG	10863920	1,2	YEEIVLERGNSGLGFSIAGGIDNPHVPDDPGIFITKII PGGAAAMDGRLGVNDCVLRVNEVEVSEVVHSRA VEALKEAGPVVRLVVRRRQPPPETIMEVNLLKGPK GLGFSIAGGIGNQHIPGDNSIYITKIIEGGAAQKDGR LQIGDRLLAVNNTNLQDVRHEEAVASLKNTSDMVY LKVAKPGSL	153
Neurabin II	AJ401189	1	RVERLELFPVELEKDSEGLGISIIGMGAGADMGLEK LGIFVKTVTEGGAAHRDGRIQVNDLLVEVDGTSLV GVTQSFAASVLRNTKGRVRCRFMIGRERPGEQSE V	154
NOS1	642525	1	QPNVISVRLFKRKVGGLGFLVKERVSKPPVIISDLIR GGAAEQSGLIQAGDIILAVNGRPLVDLSYDSALEVL RGIASETHVVLILRGPE	155
novel PDZ gene	7228177	2	PSDTSSEDGVRRIVHLYTTSDDFCLGFNIRGGKEF GLGIYVSKVDHGGLAEENGIKVGDQVLAANGVRFD DISHSQAVEVLKGQTHIMLTIKETGRYPAYKEM	156
novel PDZ gene	7228177	1	EANSDESDIIHSVRVEKSPAGRLGFSVRGGSEHGL GIFVSKVEEGSSAERAGLCVGDKITEVNGLSLESTT MGSAVKVLTSSSRLHMMVRRMGRVPGIKFSKEK	157
novel serine protease	1621243	1	DKIKKFLTESHDRQAKGKAITKKKYIGIRMMSLTSS KAKELKDRHRDFPDVISGAYIIEVIPDTPAEAGGLK ENDVIISINGQSVVSANDVSDVIKRESTLNMVVRRG NEDIMITV	158
Numb BP	AK056823	2	YRPRDDSFHVILNKSSPEEQLGIKLVRKVDEPGVFI FNALDGGVAYRHGQLEENDRVLAINGHDLRYGSP ESAAHLIQASERRVHLVVSRQVRQRSPD	159
Numb BP	AK056823	3	PTITCHEKVVNIQKDPGESLGMTVAGGASHREWD LPIYVISVEPGGVISRDGRIKTGDILLNVDGVELTEV SRSEAVALLKRTSSSIVLKALEVKEYEPQ	160
Numb BP	AK056823	1	PDGEITSIKINRVDPSESLSIRLVGGSETPLVHIIIQHI YRDGVIARDGRLLPRDIILKVNGMDISNVPHNYAVR LLRQPCQVLWLTVMREQKFRSR	161
Numb BP	AK056823	4	PRCLYNCKDIVLRRNTAGSLGFCIVGGYEEYNGNK PFFIKSIVEGTPAYNDGRIRCGDILLAVNGRSTSGMI HACLARLLKELKGRITLTIVSWPGTFL	162
outer membrane	7023825	1	LLTEEEINLTRGPSGLGFNIVGGTDQQYVSNDSGIY VSRIKENGAAALDGRLQEGDKILSVNGQDLKNLLH QDAVDLFRNAGYAVSLRVQHRLQVQNGIHS	163
p55T	12733367	1	PVDAIRILGIHKRAGEPLGVTFRVENNDLVIARILHG GMIDRQGLLHVGDIIKEVNGHEVGNNPKELQELLK NISGSVTLKILPSYRDTITPQQ	164
PAR3	8037914	2	GKRLNIQLKKGTEGLGFSITSRDVTIGGSAPIYVKNI LPRGAAIQDGRLKAGDRLIEVNGVDLVGKSQEEVV SLLRSTKMEGTVSLLVFRQEDA	165

PAR3	8037914	1	IPNFSLDDMVKLVEVPNDGGPLGIHVVPFSARGGR TLGLLVKRLEKGGKAEHENLFRENDCIVRINDGDL RNRRFEQAQHMFRQAMRTPIIWFHVVPAANKEQY EQ	166
PAR3	8037914	3	PREFLTFEVPLNDSGSAGLGVSVKGNRSKENHAD LGIFVKSIINGGAASKDGRLRVNDQLIAVNGESLLG KTNQDAMETLRRSMSTEGNKRGMIQLIVASRISKC NELKSNSS	167
PAR3L	18874467	2	ISNKNAKKIKIDLKKGPEGLGFTVVTRDSSIHGPGPI FVKNILPKGAAIKDGRLQSGDRILEVNGRDVTGRT QEELVAMLRSTKQGETASLVIARQEGH	168
PAR3L	18874467	3	ITSEQLTFEIPLNDSGSAGLGVSLKGNKSRETGTDL GIFIKSIIHGGAAFKDGRLRMNDQLIAVNGESLLGK SNHEAMETLRRSMSMEGNIRGMIQLVILRRPERP	169
PAR3L	18874467	1	IPRTKDTLSDMTRTVEISGEGGPLGIHVVPFFSSLS GRILGLFIRGIEDNSRSKREGLFHENECIVKINNVDL VDKTFAQAQDVFRQAMKSPSVLLHVLPPQNR	170
PAR6	2613011	1	PETHRRVRLHKHGSDRPLGFYIRDGMSVRVAPQG LERVPGIFISRLVRGGLAESTGLLAVSDEILEVNGIE VAGKTLDQVTDMMVANSHNLIVTVKPANQRNNV	171
PAR6 beta	1353716	1	IPVSSIIDVDILPETHRRVRLYKYGTEKPLGFYIRDG SSVRVTPHGLEKVPGIFISRLVPGGLAQSTGLLAVN DEVLEVNGIEVSGKSLDQVTDMMIANSRNLIITVRP ANQRNNRIHRD	172
PAR6 GAMMA	13537118	1	IDVDLVPETHRRVRLHRHGCEKPLGFYIRDGASVR VTPHGLEKVPGIFISRMVPGGLAESTGLLAVNDEVL EVNGIEVAGKTLDQVTDMMIANSHNLIVTVKPANQ RNNVV	173
PDZ-73	5031978	3	PEQIMGKDVRLLRIKKEGSLDLALEGGVDSPIGKVV VSAVYERGAAERHGGIVKGDEIMAINGKIVTDYTLA EADAALQKAWNQGGDWIDLVVAVCPPKEYDD	174
PDZ-73	5031978	2	IPGNRENKEKKVFISLVGSRGLGCSISSGPIQKPGIF ISHVKPGSLSAEVGLEIGDQIVEVNGVDFSNLDHKE AVNVLKSSRSLTISIVAAAGRELFMTDEF	175
PDZ-73	5031978	1	RSRKLKEVRLDRLHPEGLGLSVRGGLEFGCGLFIS HLIKGGQADSVGLQVGDEIVRINGYSISSCTHEEVI NLIRTKKTVSIKVRHIGLIPVKSSPDEFH	176
PDZK1	2944188	2	RLCYLVKEGGSYGFSLKTVQGKKGVYMTDITPQG VAMRAGVLADDHLIEVNGENVEDASHEEVVEKVK KSGSRVMFLLVDKETDKREFIVTD	177
PDZK1	2944188	3	QFKRETASLKLLPHQPRIVEMKKGSNGYGFYLRAG SEQKGQIIKDIDSGSPAEEAGLKNNDLVVAVNGES VETLDHDSVVEMIRKGGDQTSLLVVDKETDNMYR LAEFIVTD	178
PDZK1	2944188	2,3,4	RLCYLVKEGGSYGFSLKTVQGKKGVYMTDITPQG VAMRAGVLADDHLIEVNGENVEDASHEKVVEKVK KSGSRVMFLLVDKETDKRHVEQKIQFKRETASLKL LPHQPRIVEMKKGSNGYGFYLRAGSEQKGQIIKDI DSGSPAEEAGLKNNDLVVAVNGESVETLDHDSVV EMIRKGGDQTSLLVVDKETDNMYRLAHFSPFLYYQ SQELPNGSVKEAPAPTPTSLEVSSPPDTTEEVDHK PKLCRLAKGENGYGFHLNAIRGLPGSFIKEVQKGG PADLAGLEDEDVIIEVNGVNVLDEPYEKVVDRIQSS GKNVTLLVCGK	179

PDZK1	2944188	4	PDTTEEVDHKPKLCRLAKGENGYGFHLNAIRGLPG SFIKEVQKGGPADLAGLEDEDVIIEVNGVNVLDEPY EKVVDRIQSSGKNVTLLVGKNSS	180
PDZK1	2944188	1	LTSTFNPRECKLSKQEGQNYGFFLRIEKDTEGHLV RVVEKCSPAEKAGLQDGDRVLRINGVFVDKEEHM QVVDLVRKSGNSVTLLVLDGDSYEKAGSHEPS	181
PICK1	4678411	1	LGIPTVPGKVTLQKDAQNLIGISIGGGAQYCPCLYIV QVFDNTPAALDGTVAAGDEITGVNGRSIKGKTKVE VAKMIQEVKGEVTIHYNKLQADPKQGM	182
PIST	98374330	1	SQGVGPIRKVLLLKEDHEGLGISITGGKEHGVPILIS EIHPGQPADRCGGLHVGDAILAVNGVNLRDTKHKE AVTILSQQRGEIEFEVVYVAPEVDSD	183
prIL16	1478492	2	TAEATVCTVTLEKMSAGLGFSLEGGKGSLHGDKP LTINRIFKGAASEQSETVQPGDEILQLGGTAMQGLT RFEAWNIIKALPDGPVTIVIRRKSLQSK	184
prIL16	1478492	1	IHVTILHKEEGAGLGFSLAGGADLENKVITVHRVFP NGLASQEGTIQKGNEVLSINGKSLKGTTHHDALAIL RQAREPRQAVIVTRKLTPEEFIVTD	185
prIL16	1478492	1,2	IHVTILHKEEGAGLGFSLAGGADLENKVITVHRVFP NGLASQEGTIQKGNEVLSINGKSLKGTTHHDALAIL RQAREPRQAVIVTRKLTPEAMPDLNSSTDSAASAS AASDVSVESTAEATVCTVTLEKMSAGLGFSLEGGK GSLHGDKPLTINRIFKGAASEQSETVQPGDEILQLG GTAMQGLTRFEAWNIIKALPDGPVTIVIRRKSLQSK	186
PSAP	6409315	1	IREAKYSGVLSSIGKIFKEEGLLGFFVGLIPHLLGDV VFLWGCNLLAHFINAYLVDDSVSDTPGGLGNDQN PGSQFSQALAIRSYTKFVMGIAVSMLTYPFLLVGDL MAVNNCGLQAGLPPYSPVFKSWIHCWKYLSVQG QLFRGSSLLFRRVSSGSCFALE	187
PSD95	3318652	1,2,3	EGEMEYEEITLERGNSGLGFSIAGGTDNPHIGDDP SIFITKIIPGGAAAQDGRLRVNDSILFVNEVDVREVT HSAAVEALKEAGSIVRLYVMRRKPPAEKVMEIKLIK GPKGLGFSIAGGVGNQHIPGDNSIYVTKIIEGGAAH KDGRLQIGDKILAVNSVGLEDVMHEDAVAALKNTY DVVYLKVAKPSNAYLSDSYAPPDITTSYSQHLDNEI SHSSYLGTDYPTAMTPTSPRRYSPVAKDLLGEEDI PREPRRIVIHRGSTGLGFNIVGGEDGEGIFISFILAG GPADLSGELRKGDQILSVNGVDLRNASHEQAAIAL KNAGQTVTIIAQYKPE	188
PSD95	3318652	2	HVMRRKPPAEKVMEIKLIKGPKGLGFSIAGGVGNQ HIPGDNSIYVTKIIEGGAAHKDGRLQIGDKILAVNSV GLEDVMHEDAVAALKNTYDVVYLKVAKPSNAYL	189
PSD95	3318652	3	REDIPREPRRIVIHRGSTGLGFNIVGGEDGEGIFISF ILAGGPADLSGELRKGDQILSVNGVDLRNASHEQA AIALKNAGQTVTIIAQYKPEFIVTD	190
PSD95	3318652	1	LEYEeITLERGNSGLGFSIAGGTDNPHIGDDPSIFIT KIIPGGAAAQDGRLRVNDSILFVNEVDVREVTHSAA VEALKEAGSIVRLYVMRRKPPAENSS	191
PSMD9	9184389	1	RDMAEAHKEAMSRKLGQSESQGPPRAFAKVNSIS PGSPASIAGLQVDDEIVEFGSVNTQNFQSLHNIGS VVQHSEGALAPTILLSVSM	192
PTN-3	179912	1	QNDNGDSYLVLIRITPDEDGKFGFNLKGGVDQKM PLVVSRINPESPADTCIPKLNEGDQIVLINGRDISEH THDQVVMFIKASRESHSRELALVIRRRAVRS	193

PTN-4	190747	1	IRMKPDENGRFGFNVKGGYDQKMPVIVSRVAPGT PADLCVPRLNEGDQVVLINGRDIAEHTHDQVVLFIK ASCERHSGELMLLVRPNA	194
PTPL1	515030	2	GDIFEVELAKNDNSLGISVTGGVNTSVRHGGIYVK AVIPQGAAESDGRIHKGDRVLAVNGVSLEGATHKQ AVETLRNTGQVVHLLLEKGQSPTSK	195
PTPL1	515030	1	PEREITLVNLKKDAKYGLGFQIIGGEKMGRLDLGIFI SSVAPGGPADFHGCLKPGDRLISVNSVSLEGVSH HAAIEILQNAPEDVTLVISQPKEKISKVPSTPVHL	196
PTPL1	515030	4	ELEVELLITLIKSEKASLGFTVTKGNQRIGCYVHDVI QDPAKSDGRLKPGDRLIKVNDTDVTNMTHTDAVN LLRAASKTVRLVIGRVLELPRIPMLPH	197
PTPL1	515030	3	TEENTFEVKLFKNSSGLGFSFSREDNLIPEQINASI VRVKKLFAGQPAAESGKIDVGDVILKVNGASLKGL SQQEVISALRGTAPEVFLLLCRPPPGVLPEIDT	198
PTPL1	515030	5	MLPHLLPDITLTCNKEELGFSLCGGHDSLYQVVYIS DINPRSVAAIEGNLQLLDVIHYVNGVSTQGMTLEEV NRALDMSLPSLVLKATRNDLPV	199
RGS 3	18644735	1	VCSERRYRQITIPRGKDGFGFTICCDSPVRVQAVD SGGPAERAGLQQLDTVLQLNERPVEHWKCVELAH EIRSCPSEIILLVWRMVPQVKPG	200
RGS12	3290015	1	RPSPPRVRSVEVARGRAGYGFTLSGQAPCVLSCV MRGSPADFVGLRAGDQILAVNEINVKKASHEDVVK LIGKCSGVLHMVIAEGVGRFESCS	201
Rho-GAP 10	50345878	1	SEDETFSWPGPKTVTLKRTSQGFGFTLRHFIVYPP ESAIQFSYKDEENGNRGGKQRNRLEPMDTIFVKQ VKEGGPAFEAGLCTGDRIIKVNGESVIGKTYSQVIA LIQNSDTTLELSVMPKDED	202
Rhophilin	AY082588	1	SAKNRWRLVGPVHLTRGEGGFGLTLRGDSPVLIA AVIPGSQAAAAGLKEGDYIVSVNGQPCRWWRHAE VVTELKAAGEAGASLQVVSLLPSSRLPS	203
Rhophilin-like	AF268032	1	ISFSANKRWTPPRSIRFTAEEGDLGFTLRGNAPVQ VHFLDPYCSASVAGAREGDYIVSIQLVDCKWLTLS EVMKLLKSFGEDEIEMKVVSLLDSTSSMHNKSAT	204
RIM2	12734165	1	TLNEEHSHSDKHPVTWQPSKDGDRLIGRILLNKRL KDGSVPRDSGAMLGLKVVGGKMTESGRLCAFITK VKKGSLADTVGHLRPGDEVLEWNGRLLQGATFEE VYNIILESKPEPQVELVVSRPIG	205
SEMCAP 3	5889526	2	QEMDREELELEEVDLYRMNSQDKLGLTVCYRTDD EDDIGIYISEIDPNSIAAKDGRIREGDRIIQINGIEVQN REEAVALLTSEENKNFSLLIARPELQLD	206
SEMCAP 3	5889526	1	QGEETKSLTLVLHRDSGSLGFNIIGGRPSVDNHDG SSSEGIFVSKIVDSGPAAKEGGLQIHDRIIEVNGRD LSRATHDQAVEAFKTAKEPIVVQVLRRTPRTKMFT P	207
semcap2	7019938	1	ILAHVKGIEKEVNVYKSEDSLGLTITDNGVGYAFIKR IKDGGVIDSVKTICVGDHIESINGENIVGWRHYDVA KKLKELKKEELFTMKLIEPKKAFEI	208
serine protease	2738914	1	RGEKKNSSSGISGSQRRYIGVMMLTLSPSILAELQL REPSFPDVQHGVLIHKVILGSPAHRAGLRPGDVILA IGEQMVQNAEDVYEAVRTQSQLAVQIRRGRETLTL YV	209
Shank 1	6049185	1	ILEEKTVVLQKKDNEGFGFVLRGAKADTPIEEFTPT PAFPALQYLESVDEGGVAWQAGLRTGDFLIEVNN ENVVKVGHRQVVNMIRQGGNHLVLKVVTVTRNLD	210

PDDNSS

Shank 2	7025450	1	ILKEKTVLLQKKDSEGFGFVLRGAKAQTPIEEFTPT PAFPALQYLESVDEGGVAWRAGLRMGDFLIEVNG QNVVKVGHRQVVNMIRQGGNTLMVKVVMVTRHP DMDEAVQNSS	211
Shank 3	*	1	SDYVIDDKVAVLQKRDHEGFGFVLRGAKAETPIEE FTPTPAFPALQYLESVDVEGVAWRAGLRTGDFLIE VNGVNVVKVGHKQVVALIRQGGNRLVMKVVSVTR KPEEDG	212
sim to lig of numb px2	22477649	2	SNSPREEIFQVALHKRDSGEQLGIKLVRRTDEPGV FILDLLEGGLAAQDGRLSSNDRVLAINGHDLKYGT PELAAQIIQASGERVNLTIARPGKPQPG	213
sim to lig of numb px2	22477649	3	IQCVTCQEKHITVKKEPHESLGMTVAGGRGSKSG ELPIFVTSVPPHGCLARDGRIKRGDVLLNINGIDLTN LSHSEAVAMLKASAASPAVALKALEVQIVEEAT	214
Similar to GRASP65	14286261	1	MGLGVSAEQPAGGAEGFHLHGVQENSPAQQAGL EPYFDFIITIGHSRLNKENDTLKALLKANVEKPVKLE VFNMKTMRVREVEVVPSNMWGGQGLLGASVRFC SFRRASE	215
Similar to GRASP65	14286261	2	RASEQVWHVLDVEPSSPAALAGLRPYTDYVVGSD QILQESEDFFTLIESHEGKPLKLMVYNSKSDSCRES GMWHWLWVSTPDPNSAPQLPQEATWHPTTFCST TWCPTT	216
Similar to Protein-Tyrosine-Phosphatase Homolog	21595065	1	ISVTDGPKFEVKLKKNANGLGFSFVQMEKESCSHL KSDLVRIKRLFPGQPAEENGAIAAGDIILAVNGRST EGLIFQEVLHLLRGAPQEVTLLLCRPPPGA	217
SIP1	2047327	1	QPEPLRPRLCRLVRGEQGYGFHLHGEKGRRGQFI RRVEPGSPAEAAALRAGDRLVEVNGVNVEGETHH QVVQRIKAVEGQTRLLVVDQETDEELRRRNSS	218
SIP1	2047327	2	PLRELRPRLCHLRKGPQGYGFNLHSDKSRPGQYI RSVDPGSPAARSGLRAQDRLIEVNGQNVEGLRHA EVVASIKAREDEARLLVVDPETDEHFKRNSS	219
SITAC 18	8886071	1	PGVREIHLCKDERGKTGLRLRKVDQGLFVQLVQA NTPASLVGLRFGDQLLQIDGRDCAGWSSHKAHQV VKKASGDKIVVVVRDRPFQRTVTM	220
SITAC 18	8886071	2	PFQRTVTMHKDSMGHVGFVIKKGKIVSLVKGSSAA RNGLLTNHYVCEVDGQNVIGLKDKKIMEILATAGN VVTLTIIPSVIYEHIVEFIV	221
SNPC IIa	20809633	1	SLERPRFCLLSKEEGKSFGFHLQQELGRAGHVVC RVDPGTSAQRQGLQEGDRILAVNNDVVEHEDYAV VVRRIRASSPRVLLTVLARHAHDVARAQ	222
SYNTENIN	2795862	2	LRDRPFERTITMHKDSTGHVGFIFKNGKITSIVKDS SAARNGLLTEHNICEINGQNVIGLKDSQIADILSTSG TVVTITIMPAFIFEHMNSS	223
SYNTENIN	2795862	1	LEIKQGIREVILCKDQDGKIGLRLKSIDNGIFVQLVQ ANSPASLVGLRFGDQVLQINGENCAGWSSDKAHK VLKQAFGEKITMRIHRD	224
Syntrophin 1 alpha	1145727	1	QRRRVTVRKADAGGLGISIKGGRENKMPILISKIFK GLAADQTEALFVGDAILSVNGEDLSSATHDEAVQV LKKTGKEVVLEVKYMKDVSPYFK	225
Syntrophin beta 2	476700	1	PVRRVRVVKQEAGGLGISIKGGRENRMPILISKIFP GLAADQSRALRLGDAILSVNGTDLRQATHDQAVQ	226

			ALKRAGKEVLLEVKFIRE	
Syntrophin gamma 1	9507162	1	EPFYSGERTVTIRRQTVGGFGLSIKGGAEHNIPVV VSKISKEQRAELSGLLFIGDAILQINGINVRKCRHEE VVQVLRNAGEEVTLTVSFLKRAPAFLKL	227
Syntrophin gamma 2	9507164	1	SHQGRNRRTVTLRRQPVGGLGLSIKGGSEHNVPV VISKIFEDQAADQTGMLFVGDAVLQVNGIHVENAT HEEVVHLLRNAGDEVTITVEYLREAPAFLK	228
TAX2-like protein	3253116	1	RGETKEVEVTKTEDALGLTITDNGAGYAFIKRIKEG SIINRIEAVCVGDSIEAINDHSIVGCRHYEVAKMLRE LPKSQPFTLRLVQPKRAF	229
TIAM1	4507500	1	HSIHIEKSDTAADTYGFSLSSVEEDGIRRLYVNSVK ETGLASKKGLKAGDEILEINNRAADALNSSMLKDFL SQPSLGLLVRTYPELE	230
TIAM2	6912703	1	PLNVYDVQLTKTGSVCDFGFAVTAQVDERQHLSRI FISDVLPDGLAYGEGLRKGNEIMTLNGEAVSDLDL KQMEALFSEKSVGLTLIARPPDTKATL	231
TIP1	2613001	1	QRVEIHKLRQGENLILGFSIGGGIDQDPSQNPFSED KTDKGIYVTRVSEGGPAEIAGLQIGDKIMQVNGWD MTMVTHDQARKRLTKRSEEVVRLLVTRQSLQK	232
TIP2	2613003	1	RKEVEVFKSEDALGLTITDNGAGYAFIKRIKEGSVID HIHLISVGDMIEAINGQSLLGCRHYEVARLLKELPR GRTFTLKLTEPRK	233
TIP33	2613007	1	HSHPRVVELPKTDEGLGFNVMGGKEQNSPIYISRII PGGVAERHGGLKRGDQLLSVNGVSVEGEHHEKA VELLKAAKDSVKLVVRYTPKVL	234
TIP43	2613011	1	LSNQKRGVKVLKQELGGLGISIKGGKENKMPILISKI FKGLAADQTQALYVGDAILSVNGADLRDATHDEAV QALKRAGKEVLLEVKYMREATPYVK	235
Unknown PDZ	22382223	1	IQRSSIKTVELIKGNLQSVGLTLRLVQSTDGYAGHVI IETVAPNSPAAIADLQRGDRLIAIGGVKITSTLQVLK LIKQAGDRVLVYYERPVGQSNQGA	236
Vartul	1469875	1	ILTLTILRQTGGLGISIAGGKGSTPYKGDDEGIFISR VSEEGPAARAGVRVGDKLLEVNGVALQGAEHHEA VEALRGAGTAVQMRVWRERMVEPENAEFIVTD	237
Vartul	1469875	4	RELCIQKAPGERLGISIRGGARGHAGNPRDPTDEG IFISKVSPTGAAGRDGRLRVGLRLLEVNQQSLLGLT HGEAVQLLRSVGDTLTVLVCDGFEASTDAALEVS	238
Vartul	1469875	3	LEGPYPVEEIRLPRAGGPLGLSIVGGSDHSSHPFG VQEPGVFISKVLPRGLAARSGLRVGDRILAVNGQD VRDATHQEAVSALLRPCLELSLLVRRDPAEFIVTD	239
Vartul	1469875	2	PLRQRHVACLARSERGLGFSIAGGKGSTPYRAGD AGIFVSRIAEGGAAHRAGTLQVGDRVLSINGVDVT EARHDHAVSLLTAASPTIALLLEREAGG	240
Vartul	1469875	1,2	TLTILRQTGGLGISIAGGKGSTPYKGDDEGIFISRVS EEGPAARAGVRVGDKLLEGIFVSRIAEGGAAHRAG TLQVGDRVLSINGVDVTEARHDHAVSLLTAASPTIA LLLERE	241
X-11 beta	3005559	2	IPPVTTVLIKRPDLKYQLGFSVQNGIICSLMRGGIAE RGGVRVGHRIIEINGQSVVATAHEKIVQALSNSVGE IHMKTMPAAMFRLLTGQENSSL	242
X-11 beta	3005559	1	IHFSNSENCKELQLEKHKGEILGVVVVESGWGSILP TVILANMMNGGPAARSGKLSIGDQIMSINGTSLVGL PLATCQGIIKGLKNQTQVKLNIVSCPPVTTVLIKRNS	243

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ZO-1	292937	1	IWEQHTVTLHRAPGFGFGIAISGGRDNPHFQSGET SIVISDVLKGGPAEGQLQENDRVAMVNGVSMDNV EHAFAVQQLRKSGKNAKITIRRKKKVQIPNSS	244
ZO-1	292937	2	ISSQPAKPTKVTLVKSRKNEEYGLRLASHIFVKEIS QDSLAARDGNIQEGDVVLKINGTVTENMSLTDAKT LIERSKGKLKMVVQRDRATLLNSS	245
ZO-1	292937	3	IRMKLVKFRKGDSVGLRLAGGNDVGIFVAGVLEDS PAAKEGLEEGDQILRVNNVDFTNIIREEAVLFLLDLP KGEEVTILAQKKKDVFSN	246
ZO-2	12734763	1	IQHTVTLHRAPGFGFGIAISGGRDNPHFQSGETSIV ISDVLKGGPAEGQLQENDRVAMVNGVSMDNVEH AFAVQQLRKSGKNAKITIRRKKKVQIPNSS	247
ZO-2	12734763	3	HAPNTKMVRFKKGDSVGLRLAGGNDVGIFVAGIQ EGTSAEQEGLQEGDQILKVNTQDFRGLVREDAVL YLLEIPKGEMVTILAQSRADVY	248
ZO-2	12734763	2	RVLLMKSRANEEYGLRLGSQIFVKEMTRTGLATKD GNLHEGDIILKINGTVTENMSLTDARKLIEKSRGKL QLVVLRDS	249
ZO-3	10092690	3	RGYSPDTRVVRFLKGKSIGLRLAGGNDVGIFVSGV QAGSPADGQGIQEGDQILQVNDVPFQNLTREEAV QFLLGLPPGEEMELVTQRKQDIFWKMVQSEFIVTD	250
ZO-3	10092690	1	IPGNSTIWEQHTATLSKDPRRGFGIAISGGRDRPG GSMVVSDVVPGGPAEGRLQTGDHIVMVNGVSME NATSAFAIQILKTCTKMANITVKRPRRIHLPAEFIVT D	251
ZO-3	10092690	2	QDVQMKPVKSVLVKRRDSEEFGVKLGSQIFIKHIT DSGLAARHRGLQEGDLILQINGVSSQNLSLNDTRR LIEKSEGKLSLLVLRDRGQFLVNIPNSS	252

*: No GI number for this PDZ domain containing protein - it was computer cloned at Arbor Vita Corpration. using rat Shank3 seq against human genomic clone AC000036. In silico spliced together nt6400-6496, 6985-7109, 7211-7400 to create hypothetical human Shank3.

As discussed in detail herein, the PDZ proteins listed in **TABLE 5** are naturally occurring proteins containing a PDZ domain. Thus, one aspect of the present invention is directed to the detection and modulation of interactions between a PDZ protein and PL protein pair listed in **TABLE 6** or **7 or 8**. As used herein the phrase "protein pair" or 'protein binding pair" when used in reference to a PDZ protein and PL protein refers to a PL protein and PDZ protein such as those listed in **TABLES 6** or **7** or 8 which bind to one another. It should be understood that **TABLES 6, 7, and 8** are set up to show that certain PL proteins bind to a plurality of PDZ proteins.

The interactions like those summarized in **TABLES 6, 7, and 8** can occur in a wide variety of cell types. Examples of such cells include neuronal, hematopoietic, stem, muscle, epidermal, epithelial, endothelial, and cells from essentially any tissue such as liver, lung, placenta, uterus, kidney, ovaries, testes, stomach, colon and intestine. Because the interactions disclosed herein can occur in such a wide variety of cell types, these interactions can also play an important role in a variety of biological functions.

Thus, for example, in certain embodiments of the invention, the PL proteins and/or the PDZ protein to which it binds are expressed in the nervous system (e.g., neurons). In an embodiment, the PL proteins of the invention bind a PDZ protein that is expressed in neurons. In various embodiments, the PL protein is highly expressed in neuronal cells. In still other instances the PL proteins and/or the PDZ protein to which it binds are expressed in non-neural cells (e.g., in hematopoietic cells).

In various embodiments of the invention, the PL protein is expressed or up-regulated upon cell activation (e.g., in stimulated neurons), upon entry into mitosis (e.g., up-regulation in rapidly proliferating cell populations), or in association with cell death.

A. Detection of PDZ Domain-Containing Proteins

As noted *supra*, the present inventors have identified a number of PDZ protein and NMDAR PL protein interactions and a number of PDZ protein and TRP PL protein interactions that can play a role in modulation of a number of biological functions in a variety of cell types. A comprehensive list of PDZ domain-containing proteins was retrieved from the Sanger Centre database (Pfam) searching for the keyword, "PDZ". The corresponding cDNA sequences were retrieved from GenBank using the NCBI "entrez" database (hereinafter, "GenBank PDZ protein cDNA sequences"). The DNA portion encoding PDZ domains was identified by alignment of cDNA and protein sequence using CLUSTALW. Based on the DNA/protein alignment information, primers encompassing the PDZ domains were designed. The expression of certain PDZ-containing proteins in cells was detected by polymerase chain reaction ("PCR") amplification of cDNAs obtained by reverse transcription ("RT") of cell-derived RNA (i.e., "RT-PCR"). PCR, RT-PCR and other methods for analysis and manipulation of nucleic acids are well known and are described generally in Sambrook et al., (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Vols. 1-3, Cold Spring Harbor Laboratory

hereinafter, "Sambrook"); and Ausubel et al., *Current Protocols in Molecular Biology*, Greene Publishing and Wiley-Interscience, New York (1997), as supplemented through January 1999 (hereinafter "Ausubel").

Samples of cDNA for those sequences identified through the foregoing search were obtained and then amplified. In general a sample of the cDNA (typically, 1/5 of a 20 µl reaction) was used to conduct PCR. PCR was conducted using primers designed to amplify specifically PDZ domain-containing regions of PDZ proteins of interest. Oligonucleotide primers were designed to amplify one or more PDZ-encoding domains. The DNA sequences encoding the various PDZ domains of interest were identified by inspection (i.e., conceptual translation of the PDZ protein cDNA sequences obtained from GenBank, followed by alignment with the PDZ domain amino acid sequence). **TABLE 5** shows the PDZ-encoded domains amplified, and the GenBank accession number of the polynucleotides encoding the PDZ-domain containing proteins. To facilitate subsequent cloning of PDZ domains, the PCR primers included endonuclease restriction sequences at their ends to allow ligation with pGEX-3X cloning vector (Pharmacia, GenBank XXI13852) in frame with glutathione-S transferase (GST).

TABLE 5 lists the proteins isolated for use in the aforementioned assays.

B. Production of Fusion Proteins Containing PDZ-Domains

GST-PDZ domain fusion proteins were prepared for use in the assays of the invention. PCR products containing PDZ encoding domains (as described *supra*) were subcloned into an expression vector to permit expression of fusion proteins containing a PDZ domain and a heterologous domain (i.e., a glutathione-S transferase sequence, "GST"). PCR products (i.e., DNA fragments) representing PDZ domain encoding DNA was extracted from agarose gels using the "sephaglas" gel extraction system (Pharmacia) according to the manufacturer's recommendations.

As noted *supra*, PCR primers were designed to include endonuclease restriction sites to facilitate ligation of PCR fragments into a GST gene fusion vector (pGEX-3X; Pharmacia, GenBank accession # XXU13852) in-frame with the glutathione-S transferase coding sequence. This vector contains a IPTG inducible lacZ promoter. The pGEX-3X vector was linearized using *Bam* HI and *Eco* RI or, in some cases, *Eco* RI or *Sma* I, and dephosphorylated. For most cloning approaches, double digestion with Bam HI and Eco RI was performed, so that

the ends of the PCR fragments to clone were Bam HI and Eco RI. In some cases, restriction endonuclease combinations used were Bgl II and Eco RI, Bam HI and Mfe I, or Eco RI only, Sma I only, or BamHI only. When more than one PDZ domain was cloned, the DNA portion cloned represents the PDZ domains and the cDNA portion located between individual domains. DNA linker sequences between the GST portion and the PDZ domain containing DNA portion vary slightly, dependent on which of the above described cloning sites and approaches were used. As a consequence, the amino acid sequence of the GST-PDZ fusion protein varies in the linker region between GST and PDZ domain. Protein linker sequences corresponding to different cloning sites/approaches are shown below. Linker sequences (vector DNA encoded) are bold, PDZ domain containing gene derived sequences are in italics.

1) **GST—BamHI/***BamHI— PDZ domain insert*
Gly--Ile—*PDZ domain insert*

2) **GST—BamHI/***BglII—PDZ domain insert*
Gly—Ile—*PDZ domain insert*

3) **GST—EcoRI/***EcoI—PDZ domain insert*
Gly—Ile—Pro—Gly--Asn—*PDZ domain insert* (SEQ ID NO:253)

4) **GST--SmaI/***SmaI—PDZ domain insert*
Gly—Ile—Pro—*PDZ domain insert*

The PDZ-encoding PCR fragment and linearized pGEX-3X vector were ethanol precipitated and resuspended in 10 µl standard ligation buffer. Ligation was performed for 4-10 hours at 7°C using T4 DNA ligase. It will be understood that some of the resulting constructs include very short linker sequences and that, when multiple PDZ domains were cloned, the constructs included some DNA located between individual PDZ domains.

The ligation products were transformed in DH5α or BL-21 *E.coli* bacteria strains. Colonies were screened for presence and identity of the cloned PDZ domain containing DNA as well as for correct fusion with the glutathione S-transferase encoding DNA portion by PCR and by sequence analysis. Positive clones were tested in a small scale assay for expression of the

GST/PDZ domain fusion protein and, if expressing, these clones were subsequently grown up for large scale preparations of GST/PDZ fusion protein.

GST-PDZ domain fusion protein was overexpressed following addition of IPTG to the culture medium and purified. Detailed procedure of small scale and large scale fusion protein expression and purification are described in "GST Gene Fusion System" (second edition, revision 2; published by Pharmacia). In brief, a small culture (3-5ml) containing a bacterial strain (DH5α, BL21 or JM109) with the fusion protein construct was grown overnight in LB-media at 37°C with the appropriate antibiotic selection (100μg/ml ampicillin; a.k.a. LB-amp). The overnight culture was poured into a fresh preparation of LB-amp (typically 250-500 ml) and grown until the optical density (OD) of the culture was between 0.5 and 0.9 (approximately 2.5 hours). IPTG (isopropyl β-D-thiogalactopyranoside) was added to a final concentration of 1.0mM to induce production of GST fusion protein, and culture was grown an additional 1.5-2.5 hours. Bacteria were collect by centrifugation (4500 g) and resuspended in Buffer A- (50 mM Tris, pH 8.0, 50 mM dextrose, 1 mM EDTA, 200μM phenylmethylsulfonylfluoride). An equal volume of Buffer A+ (Buffer A-, 4 mg/ml lysozyme) was added and incubated on ice for 3 min to lyse bacteria. An equal volume of Buffer B (10 mM Tris, pH 8.0, 50 mM KCl, 1 mM EDTA. 0.5% Tween-20, 0.5% NP40 (a.k.a. IGEPAL CA-630), 200μM phenylmethylsulfonylfluoride) was added and incubated for an additional 20 minutes. The bacterial cell lysate was centrifuged (x20,000g), and supernatant was added to glutathione SEPHAROSE 4B beads (Pharmacia, Cat. # 17-0765-01) previously swelled (rehydrated) in 1X phosphate-buffered saline (PBS). The supernatant-glutathione SEPHAROSE bead slurry was poured into a column and washed with at least 20 bed volumes of 1X PBS. GST fusion protein was eluted off the glutathione SEPHAROSE beads by applying 0.5-1.0 ml aliquots of 5mM glutathione and collected as separate fractions. Concentrations of fractions were determined using BioRad Protein Assay (Cat. # 500-0006) according to manufacturer's specifications. Those fractions containing the highest concentration of fusion protein were pooled and dialyzed against 1X PBS/35% glycerol. Fusion proteins were assayed for size and quality by SDS gel electrophoresis (PAGE) as described in "Sambrook." Fusion protein aliquots were stored at minus 80°C and at minus 20°C.

C. Classification of PDZ Domain-Containing Proteins

The PDZ proteins identified herein as interacting with particular PL proteins can be grouped into a number of different categories. Thus, as described in greater detail below, the methods and reagents that are provided herein can be utilized to modulate PDZ interactions, and thus biological functions, that are regulated or otherwise involve the following classes of proteins. It should be recognized, however, that modulation of the interactions that are identified herein can be utilized to affect biological functions involving other protein classes.

1) Protein Kinases

A number of protein kinases contain PDZ domains. Protein kinases are widely involved in cellular metabolism and regulation of protein activity through phosphorylation of amino acids on proteins. An example of this is the regulation of signal transduction pathways such as T cell activation through the T cell Receptor, where ZAP-70 kinase function is required for transmission of the activation signal to downstream effector molecules. These molecules include, but are not limited to KIAA0303, KIAA0561, KIAA0807, KIAA0973, and CASK.

2) Guanalyte Kinases

A number of guanalyte kinases contain PDZ domains. These molecules include, but are not limited to Atrophin 1, CARD11, CARD14, DLG1, DLG2, DLG5, FLJ12615, MPP1, MPP2, NeDLG, p55T, PSD95, ZO-1, ZO-2, and ZO-3.

3) Guanine Exchange Factors

A number of guanine exchange factors contain PDZ domains. Guanine exchange factors regulate signal transduction pathways and other biological processes through facilitating the exchange of differently phosphorylated guanine residues. These molecules include, but are not limited to GTPase, Guanine Exchange, KIAA0313, KIAA0380, KIAA0382, KIAA1389, KIAA1415, TIAM1, and TIAM2.

4) LIM PDZ's

A number of LIM proteins contain PDZ domains. These molecules include, but are not limited to α-Actinin 2, ELFIN1, ENIGMA, HEMBA 1003117, KIAA0613, KIAA0858, KIAA0631, LIM Mystique, LIM protein, LIM-RIL, LIMK1, LIMK2, and LU-1.

5) Proteins Containing Only PDZ Domains

A number of proteins contain PDZ domains without any other predicted functional domains. These include, but are not limited to 26s subunit p27, AIPC, Cytohesion Binding Protein, EZRIN Binding Protein, FLJ00011, FLJ20075, FLJ21687, GRIP1, HEMBA1000505, KIAA0545, KIAA0967, KIAA1202, KIAA1284, KIAA1526, KIAA1620, KIAA1719, MAGI1, Novel PDZ gene, Outer Membrane, PAR3, PAR6, PAR6γ, PDZ-73, PDZK1, PICK1, PIST, prIL16, Shank1, SIP1, SITAC-18, Syntenin, Syntrophin γ2, TIP1, TIP2, and TIP43.

6) Tyrosine Phosphatases

A number of tyrosine phosphatases contain PDZ domains. Tyrosine phosphatases regulate biological processes such as signal transduction pathways through removal of phosphate groups required for function of the target protein. Examples of such enzymes include, but are not limited to PTN-3, PTN-4, and PTPL1.

7) Serine Proteases

A number of serine proteases contain PDZ domains. Proteases affect biological molecules by cleaving them to either activate or repress their functional ability. These enzymes have a variety of functions, including roles in digestion, blood coagulation and lysis of blood clots. These include, but are not limited to Novel Serine Protease and Serine Protease.

8) Viral Oncogene Interacting Proteins that Contain PDZ Domains

A number of TAX interacting proteins contain PDZ domains. Many of these also bind to multiple viral oncoproteins such as Adenovirus E4, Papillomavirus E6, and HBV protein X. These TAX interacting proteins include, but are not limited to AIPC, Connector Enhancer, DLG1, DLG2, ERBIN, FLJ00011, FLJ11215, HEMBA1003117, INADL, KIAA0147, KIAA0807, KIAA1526, KIAA1634, LIMK1, LIM Mystique, LIM-RIL, MUPP1, NeDLG, Outer Membrane, PSD95, PTN-4, PTPL-1, Syntrophin γ1, Syntrophin γ2, TAX2-like protein, TIP2, TIP1, TIP33, and TIP43.

9) Other PDZ-domain containing proteins

A number of proteins containing RA and/or RHA and/or DIL and/or IGFBP and/or WW and/or L27 and/or SAM and/or PH and/or DIX and/or DIP and/or Dishevelled and/or LRR and/or Hormone 3 and/or C2 and/or RPH3A and/or zf-TRAF and/or zf-C3HC4 and/or PID and/or NO_Synthase and/or Flavodoxin and/or FAD binding and/or NAD binding and/or Kazal and/or Trypsin and/or RBD and/or RGS and/or GoLoco and/or HR1 and/or BR01 domains contain PDZ domains. These include, but are not limited to AF6, APXL-1, MAGI1, DVL1, DVL2, DVL3, KIAA0417, KIAA0316, KIAA0340, KIAA0559, KIAA0751, KIAA0902, KIAA1095, KIAA1222, KIAA1634, MINT1, NOS1, RGS12, Rhophilin-like, Shank 3, Syntrophin 1α, Syntrophin β2, and X11β.

D. Assays for Detection of Interactions Between PDZ-Domain Polypeptides and TRP PL Proteins and TRP Associated Proteins

Two complementary assays, termed A and G, were developed to detect binding between a PDZ-domain polypeptide and candidate PDZ ligand. In each of the two different assays, binding is detected between a peptide having a sequence corresponding to the C-terminus of a protein anticipated to bind to one or more PDZ domains (i.e. a candidate PL peptide) and a PDZ-domain polypeptide (typically a fusion protein containing a PDZ domain). In the A assay, the candidate PL peptide is immobilized and binding of a soluble PDZ-domain polypeptide to the immobilized peptide is detected (the A assay is named for the fact that in one embodiment an avidin surface is used to immobilize the peptide). In the G assay, the PDZ-domain polypeptide is immobilized and binding of a soluble PL peptide is detected (The G assay is named for the fact that in one embodiment a GST-binding surface is used to immobilize the PDZ-domain polypeptide). Preferred embodiments of these assays are described in detail *infra*. However, it will be appreciated by ordinarily skilled practitioners that these assays can be modified in numerous ways while remaining useful for the purposes of the present invention.

1) A Assay Detection of PDZ-Ligand Binding Using Immobilized PL Peptide.

In one aspect, the invention provides an assay in which biotinylated candidate PL peptides are immobilized on an avidin coated surface. The binding of PDZ-domain fusion

protein to this surface is then measured. In a preferred embodiment, the PDZ-domain fusion protein is a GST/PDZ fusion protein and the assay is carried out as follows:

(1) Avidin is bound to a surface, e.g. a protein binding surface. In one embodiment, avidin is bound to a polystyrene 96 well plate (e.g., Nunc Polysorb (Cat. #475094) by addition of 100 µl per well of 20 µg/ml of avidin (Pierce) in phosphate buffered saline without calcium and magnesium, pH 7.4 ("PBS", GibcoBRL) at 4°C for 12 hours. The plate is then treated to block nonspecific interactions by addition of 200 µl per well of PBS containing 2 g per 100 ml protease-free bovine serum albumin ("PBS/BSA") for 2 hours at 4°C. The plate is then washed 3 times with PBS by repeatedly adding 200 µl per well of PBS to each well of the, plate and then dumping the contents of the plate into a waste container and tapping the plate gently on a dry surface.

(2) Biotinylated PL peptides (or candidate PL peptides, e.g. see **TABLE 2** and **TABLE 4**) are immobilized on the surface of wells of the plate by addition of 50 µl per well of 0.4 µM peptide in PBS/BSA for 30 minutes at 4°C. Usually, each different peptide is added to at least eight different wells so that multiple measurements (e.g. duplicates and also measurements using different (GST/PDZ-domain fusion proteins and a GST alone negative control) can be made, and also additional negative control wells are prepared in which no peptide is immobilized. Following immobilization of the PL peptide on the surface, the plate is washed 3 times with PBS.

(3) GST/PDZ-domain fusion protein (prepared as described *supra*) is allowed to react with the surface by addition of 50 µl per well of a solution containing 5 µg/ml GST/PDZ-domain fusion protein in PBS/BSA for 2 hours at 4°C. As a negative control, GST alone (i.e. not a fusion protein) is added to specified wells, generally at least 2 wells (i.e. duplicate measurements) for each immobilized peptide. After the 2 hour reaction, the plate is washed 3 times with PBS to remove unbound fusion protein.

(4) The binding of the GST/PDZ-domain fusion protein to the avidin-biotinylated peptide surface can be detected using a variety of methods, and detectors known in the art. In one embodiment, 50 µl per well of an anti-GST antibody in PBS/BSA (e.g. 2.5 µg/ml of polyclonal goat-anti-GST antibody, Pierce) is added to the plate and allowed to react for 20 minutes at 4°C. The plate is washed 3 times with PBS and a second, detectably labeled antibody

is added. In one embodiment, 50 µl per well of 2.5 µg/ml of horseradish peroxidase (HRP)-conjugated polyclonal rabbit anti-goat immunoglobulin antibody is added to the plate and allowed to react for 20 minutes at 4°C. The plate is washed 5 times with 50 mM Tris pH 8.0 containing 0.2% Tween 20, and developed by addition of 100 µl per well of HRP-substrate solution (TMB, Dako) for 20 minutes at room temperature (RT). The reaction of the HRP and its substrate is terminated by the addition of 100 µl per well of 1 M sulfuric acid and the optical density (O.D.) of each well of the plate is read at 450 nm.

(5) Specific binding of a PL peptide and a PDZ-domain polypeptide is detected by comparing the signal from the well(s) in which the PL peptide and PDZ domain polypeptide are combined with the background signal(s). The background signal is the signal found in the negative controls. Typically a specific or selective reaction will be at least twice background signal, more typically more than 5 times background, and most typically 10 or more times the background signal. In addition, a statistically significant reaction will involve multiple measurements of the reaction with the signal and the background differing by at least two standard errors, more typically four standard errors, and most typically six or more standard errors. Correspondingly, a statistical test (e.g. a T-test) comparing repeated measurements of the signal with repeated measurements of the background will result in a p-value < 0.05, more typically a p-value < 0.01, and most typically a p-value < 0.001 or less.

As noted, in an embodiment of the A assay, the signal from binding of a GST/PDZ-domain fusion protein to an avidin surface not exposed to (i.e. not covered with) the PL peptide is one suitable negative control (sometimes referred to as B1). The signal from binding of GST polypeptide alone (i.e. not a fusion protein) to an avidin-coated surface that has been exposed to (i.e. covered with) the PL peptide is a second suitable negative control (sometimes referred to as B2. Because all measurements are done in multiples (i.e. at least duplicate) the arithmetic mean (or, equivalently, average) of several measurements is used in determining the binding, and the standard error of the mean is used in determining the probable error in the measurement of the binding. The standard error of the mean of N measurements equals the square root of the following: the sum of the squares of the difference between each measurement and the mean, divided by the product of (N) and (N-1). Thus, in one embodiment, specific binding of the PDZ protein to the plate-bound PL peptide is determined by comparing

the mean signal ("mean S") and standard error of the signal ("SE") for a particular PL-PDZ combination with the mean B1 and/or mean B2.

2) G Assay-Detection of PDZ-Ligand Binding Using Immobilized PDZ-Domain Fusion Polypeptide

In one aspect, the invention provides an assay in which a GST/PDZ fusion protein is immobilized on a surface (G assay). The binding of labeled PL peptide (e.g., as listed in **TABLE 2** and **TABLE 4**) to this surface is then measured. In a preferred embodiment, the assay is carried out as follows:

(1) A PDZ-domain polypeptide is bound to a surface, e.g. a protein binding surface. In a preferred embodiment, a GST/PDZ fusion protein containing one or more PDZ domains is bound to a polystyrene 96-well plate. The GST/PDZ fusion protein can be bound to the plate by any of a variety of standard methods known to one of skill in the art, although some care must be taken that the process of binding the fusion protein to the plate does not alter the ligand-binding properties of the PDZ domain. In one embodiment, the GST/PDZ fusion protein is bound via an anti-GST antibody that is coated onto the 96-well plate. Adequate binding to the plate can be achieved when:

a. 100 µl per well of 5 µg/ml goat anti-GST polyclonal antibody (Pierce) in PBS is added to a polystyrene 96-well plate (e.g., Nunc Polysorp) at 4°C for 12 hours.

b. The plate is blocked by addition of 200 µl per well of PBS/BSA for 2 hours at 4°C.

c. The plate is washed 3 times with PBS.

d. 50 µl per well of 5 µg/ml GST/PDZ fusion protein) or, as a negative control, GST polypeptide alone (i.e. not a fusion protein) in PBS/BSA is added to the plate for 2 hours at 4°C.

e. the plate is again washed 3 times with PBS.

(2) Biotinylated PL peptides are allowed to react with the surface by addition of 50 µl per well of 20 µM solution of the biotinylated peptide in PBS/BSA for 10 minutes at 4°C, followed by an additional 20 minute incubation at 25°C. The plate is washed 3 times with ice cold PBS.

(3) The binding of the biotinylated peptide to the GST/PDZ fusion protein surface can be detected using a variety of methods and detectors known to one of skill in the art. In one embodiment, 100 µl per well of 0.5 µg/ml streptavidin-horseradish peroxidase (HRP) conjugate dissolved in BSA/PBS is added and allowed to react for 20 minutes at 4°C. The plate is then washed 5 times with 50 mM Tris pH 8.0 containing 0.2% Tween 20, and developed by addition of 100 µl per well of HRP-substrate solution (TMB, Dako) for 20 minutes at room temperature (RT). The reaction of the HRP and its substrate is terminated by addition of 100 µl per well of 1 M sulfuric acid, and the optical density (O.D.) of each well of the plate is read at 450 um.

(4) Specific binding of a PL peptide and a PDZ domain polypeptide is determined by comparing the signal from the well(s) in which the PL peptide and PDZ domain polypeptide are combined, with the background signal(s). The background signal is the signal found in the negative control(s). Typically a specific or selective reaction will be at least twice background signal, more typically more than 5 times background, and most typically 10 or more times the background signal. In addition, a statistically significant reaction will involve multiple measurements of the reaction with the signal and the background differing by at least two standard errors, more typically four standard errors, and most typically six or more standard errors. Correspondingly, a statistical test (e.g. a T-test) comparing repeated measurements of the signal with -repeated measurements of the background will result in a p-value < 0.05, more typically a p-value < 0.01, and most typically a p-value < 0.001 or less. As noted, in an embodiment of the G assay, the signal from binding of a given PL peptide to immobilized (surface bound) GST polypeptide alone is one suitable negative control (sometimes referred to as B1). Because all measurement are done in multiples (i.e. at least duplicate) the arithmetic mean (or, equivalently, average.) of several measurements is used in determining the binding, and the standard error of the mean is used in determining the probable error in the measurement of the binding. The standard error of the mean of N measurements equals the square root of the following: the sum of the squares of the difference between each measurement and the mean, divided by the product of (N) and (N-1). Thus, in one embodiment, specific binding of the PDZ protein to the platebound peptide is determined by comparing the mean signal ("mean S") and standard error of the signal ("SE") for a particular PL-PDZ combination with the mean B1.

i) G1 assay, G2 assay, and G3 assay

Three specific modifications of the specific conditions described *supra* for the G assay are particularly useful. The modified assays use lesser quantities of labeled PL peptide and have slightly different biochemical requirements for detection of PDZ-ligand binding compared to the specific assay conditions described *supra*. For convenience, the assay conditions described in this section are referred to as the G1 assay, the G2 assay, and the G3 assay, with the specific conditions described in the preceding section on G assays being referred to as the G0 assay. The G1 assay is identical to the G0 assay except at step (2) the peptide concentration is 10 μM instead of 20 μM. This results in slightly lower sensitivity for detection of interactions with low affinity and/or rapid dissociation rate. Correspondingly, it slightly increases the certainty that detected interactions are of sufficient affinity and half-life to be of biological importance and useful therapeutic targets.

The G2 assay is identical to the G0 assay except that at step (2) the peptide concentration is 1 μM instead of 20 μM and the incubation is performed for 60 minutes at 25°C (rather than, e.g., 10 minutes at 4°C followed by 20 minutes at 25°C). This results in lower sensitivity for interactions of low affinity, rapid dissociation rate, and/or affinity that is less at 25°C than at 4°C. Interactions will have lower affinity at 25°C than at 4°C if (as we have found to be generally true for PDZ-ligand binding) the reaction entropy is negative (i.e. the entropy of the products is less than the entropy of the reactants). In contrast, the PDZ-PL binding signal may be similar in the G2 assay and the G0 assay for interactions of slow association and dissociation rate, as the PDZ-PL complex will accumulate during the longer incubation of the "G'' assay." Thus comparison of results of the G2 assay and the G0 assay can be used to estimate the relative entropies, enthalpies, and kinetics of different PDZ-PL interactions. (Entropies and enthalpies are related to binding affinity by the equations delta G = RT ln (Kd) = delta H – T delta S where delta G, H, and S are the reaction free energy, enthalpy, and entropy respectively, T is the temperature in degrees Kelvin, R is the gas constant, and Kd is the equilibrium dissociation constant). In particular, interactions that are detected only or much more strongly in the G0 assay generally have a rapid dissociation rate at 25°C ($t_{1/2}$ < 10 minutes) and a negative reaction entropy, while interactions that are detected similarly strongly in the "G'' assay" generally have a slower dissociation rate at 25°C ($t_{1/2}$ > 10 minutes). Rough estimation of the thermodynamics and kinetics of PDZ-PL interactions (as can be achieved via

comparison of results of the G0 assay versus the G2 assay as outlined *supra)* can be used in the design of efficient inhibitors of the interactions. For example, a small molecule inhibitor based on the chemical structure of a PL that dissociates slowly from a given PDZ domain (as evidenced by similar binding in the G2 assay as in the G0 assay) may itself dissociate slowly and thus be of high affinity.

The G3 assay is identical to the G0 assay with the following exceptions. The peptides are typically present at 0.1 μM rather than 20 μM. The peptides are also pre-incubated with the HRP-streptavidin prior to adding to the assay plate. In the G0 assay, free peptide is incubated with the PDZ proteins prior to the addition of the HRP--streptavidin. Thus, for the G0 assay one can lose signal if the bound peptide dissociates from the PDZ protein prior to the addition of the HRP-streptavidin. In the G3 modified assay the HRP-streptavidin/peptide complex is added to the plate in one step, thus increasing the likelihood that all the bound peptide will be bound to HRP-streptavidin. The G3 modified assay increases the chance of observing weak interaactions.

In this manner, variation of the temperature and duration of step (2) of the G assay can be used to provide insight into the kinetics and thermodynamics of the PDZ-ligand binding reaction and into design of inhibitors of the reaction.

With any of the assays, peptides should be titrated to find the optimal concentration for which the signal to noise ratio is in the appropriate range over the entire collection of PDZ domains tested.

3) Assay Variations

As discussed *supra*, it will be appreciated that many of the steps in the above-described assays can be varied, for example, various substrates can be used for binding the PL and PDZ-containing proteins; different types of PDZ containing fusion proteins can be used; different labels for detecting PDZ/PL interactions can be employed; and different ways of detection can be used.

The PL protein used in the assay is not intended to be limited to a 20 amino acid peptide. Full length or partial protein may be used, either alone or as a fusion protein. For

example, a GST-PL protein fusion may be bound to the anti-GST antibody, with PDZ protein added to the bound PL protein or peptide.

The PDZ-PL detection assays can employ a variety of surfaces to bind the PL and PDZ-containing proteins. For example, a surface can be an "assay plate" which is formed from a material (e.g. polystyrene) which optimizes adherence of either the PL protein or PDZ-containing protein thereto. Generally, the individual wells of the assay plate will have a high surface area to volume ratio and therefore a suitable shape is a flat bottom well (where the proteins of the assays are adherent). Other surfaces include, but are not limited to, polystyrene or glass beads, polystyrene or glass slides, and the like.

For example, the assay plate can be a multiwell plate. The term multiwell plate when used herein refers to a multiwell assay plate, e.g., having between about 30 to 200 individual wells, usually 96 wells. Alternatively, high density arrays can be used. Often, the individual wells of the multiwell plate will hold a maximum volume of about 250 µl. Conveniently, the assay plate is a 96 well polystyrene plate (such as that sold by Becton Dickinson Labware, Lincoln Park, N.J.), which allows for automation and high throughput screening. Other surfaces include polystyrene multiwell ELISA plates such as that sold by Nunc Maxisorp, Inter Med, Denmark. Often, about 50 µl to 300 µl, more preferably 100 µl to 200 µl, of an aqueous sample comprising buffers suspended therein will be added to each well of the assay plate.

The detectable labels of the invention can be any detectable compound or composition which is conjugated directly or indirectly with a molecule (such as described above). The label can be detectable by itself (e.g., radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, can catalyze a chemical alteration of a substrate compound or composition which is detectable. The preferred label is an enzymatic one which catalyzes a color change of a non-radioactive color reagent.

Sometimes, the label is indirectly conjugated with the antibody. One of skill is aware of various techniques for indirect conjugation. For example, the antibody can be conjugated with biotin and any of the categories of labels mentioned above can be conjugated with avidin, or vice versa (see also A and G assay above). Biotin binds selectively to avidin and thus, the label can be conjugated with the antibody in this indirect manner. See, Ausubel, *supra*, for a review of techniques involving biotin-avidin conjugation and similar assays. Alternatively, to achieve indirect conjugation of the label with the antibody, the antibody is conjugated with a

small hapten (e.g. digoxin) and one of the different types of labels mentioned above is conjugated with an anti-hapten antibody (e.g. anti-digoxin antibody). Thus, indirect conjugation of the label with the antibody can be achieved.

Assay variations can include different washing steps. By "washing" is meant exposing the solid phase to an aqueous solution (usually a buffer or cell culture media) in such a way that unbound material (e.g., non-adhering cells, non-adhering capture agent, unbound ligand, receptor, receptor construct, cell lysate, or HRP antibody) is removed therefrom. To reduce background noise, it is convenient to include a detergent (e.g., Triton X) in the washing solution. Usually, the aqueous washing solution is decanted from the wells of the assay plate following washing. Conveniently, washing can be achieved using an automated washing device. Sometimes, several washing steps (e.g., between about 1 to 10 washing steps) can be required.

Various buffers can also be used in PDZ-PL detection assays. For example, various blocking buffers can be used to reduce assay background. The term "blocking buffer" refers to an aqueous, pH buffered solution containing at least one blocking compound which is able to bind to exposed surfaces of the substrate which are not coated with a PL or PDZ-containing protein. The blocking compound is normally a protein such as bovine serum albumin (BSA), gelatin, casein or milk powder and does not cross-react with any of the reagents in the assay. The block buffer is generally provided at a pH between about 7 to 7.5 and suitable buffering agents include phosphate and TRIS.

Various enzyme-substrate combinations can also be utilized in detecting PDZ-PL interactions. Examples of enzyme-substrate combinations include, for example:

(i) Horseradish peroxidase (HRPO) with hydrogen peroxidase as a substrate, wherein the hydrogen peroxidase oxidizes a dye precursor (e.g. orthophenylene diamine [OPD] or 3,3',5,5'-tetramethyl benzidine hydrochloride [TMB]) (as described above).

(ii) alkaline phosphatase (AP) with para-Nitrophenyl phosphate as chromogenic substrate.

(iii) ß-D-galactosidase (ß D-Gal) with a chromogenic substrate (e.g. p-nitrophenyl- ß-D-galactosidase) or fluorogenic substrate 4-methylumbelliferyl- ß-D-galactosidase.

Numerous other enzyme-substrate combinations are available to those skilled in the art. For a general review of these, see U.S. Patent Nos. 4,275,149 and 4,318,980, both of which are herein incorporated by reference.

Further, it will be appreciated that, although, for convenience, the present discussion primarily refers antagonists of PDZ-PL interactions, agonists of PDZ-PL interactions can be identified using the methods disclosed herein or readily apparent variations thereof.

E. Detecting PDZ-PL Interactions

The present inventors were able in part to identify the interactions summarized in **TABLES 6, 7, and 8** by developing new high throughput screening assays which are described *supra*. Various other assay formats known in the art can be used to select ligands that are specifically reactive with a particular protein. For example, solid-phase ELISA immunoassays, immunoprecipitation, Biacore, Fluorescence Polarization (FP), Fluorescence Resonance Energy Transfer (FRET) and western blot assays can be used to identify peptides that specifically bind PDZ-domain polypeptides. As discussed *supra*, two different, complementary assays were developed to detect PDZ-PL interactions. In each, one binding partner of a PDZ-PL pair is immobilized, and the ability of the second binding partner to bind is determined. These assays, which are described *supra*, can be readily used to screen for hundreds to thousands of potential PDZ-ligand interactions in a few hours. Thus these assays can be used to identify yet more novel PDZ-PL interactions in neuronal cells. In addition, they can be used to identify antagonists of PDZ-PL interactions (see *infra*).

In various embodiments, fusion proteins are used in the assays and devices of the invention. Methods for constructing and expressing fusion proteins are well known. Fusion proteins generally are described in Ausubel et al., supra, Kroll et al., 1993, DNA Cell. Biol. 12:441, and Imai et al., 1997, *Cell* 91:521-30. Usually, the fusion protein includes a domain to facilitate immobilization of the protein to a solid substrate ("an immobilization domain"). Often, the immobilization domain includes an epitope tag (i.e., a sequence recognized by a antibody, typically a monoclonal antibody) such as polyhistidine (Bush et al, 1991, *J. Biol Chem* 266:13811-14), SEAP (Berger et al, 1988, *Gene* 66:1-10), or M1 and M2 flag (see, e.g., U.S. Patent Nos. 5,011,912; 4,851,341; 4,703,004; 4,782,137). In an embodiment, the immobilization domain is a GST coding region. It will be recognized that, in addition to the PDZ-domain and

the particular residues bound by an immobilized antibody, protein A, or otherwise contacted with the surface, the protein (e.g., fusion protein), will contain additional residues. In some embodiments these are residues naturally associated with the PDZ-domain (i.e., in a particular PDZ-protein) but they may include residues of synthetic (e.g., poly(alanine)) or heterologous origin (e.g., spacers of, e.g., between 10 and 300 residues).

PDZ domain-containing polypeptide used in the methods of the invention (e.g., PDZ fusion proteins) of the invention are typically made by (1) constructing a vector (e.g., plasmid, phage or phagemid) comprising a polynucleotide sequence encoding the desired polypeptide, (2) introducing the vector into an suitable expression system (e.g., a prokaryotic, insect, mammalian, or cell free expression system), (3) expressing the fusion protein and (4) optionally purifying the fusion protein.

In one embodiment, expression of the protein comprises inserting the coding sequence into an appropriate expression vector (i.e., a vector that contains the necessary elements for the transcription and translation of the inserted coding sequence required for the expression system employed, e.g., control elements including enhancers, promoters, transcription terminators, origins of replication, a suitable initiation codon (e.g., methionine), open reading frame, and translational regulatory signals (e.g., a ribosome binding site, a termination codon and a polyadenylation sequence. Depending on the vector system and host utilized, any number of suitable transcription and translation elements, including constitutive and inducible promoters, can be used.

The coding sequence of the fusion protein includes a PDZ domain and an immobilization domain as described elsewhere herein. Polynucleotides encoding the amino acid sequence for each domain can be obtained in a variety of ways known in the art; typically the polynucleotides are obtained by PCR amplification of cloned plasmids, cDNA libraries, and cDNA generated by reverse transcription of RNA, using primers designed based on sequences determined by the practitioner or, more often, publicly available (e.g., through GenBank). The primers include linker regions (e.g., sequences including restriction sites) to facilitate cloning and manipulation in production of the fusion construct. The polynucleotides corresponding to the PDZ and immobilization regions are joined in-frame to produce the fusion protein-encoding sequence.

The fusion proteins of the invention may be expressed as secreted proteins (e.g., by including the signal sequence encoding DNA in the fusion gene; see, e.g., Lui et al, 1993, *PNAS USA*, 90:8957-61) or as nonsecreted proteins.

In some embodiments, the PDZ-containing proteins are immobilized on a solid surface. The substrate to which the polypeptide is bound may in any of a variety of forms, e.g., a multiwell dish, a test tube, a dipstick, a microcentrifuge tube, a bead, a spinnable disk, and the like. Suitable materials include glass, plastic (e.g., polyethylene, PVC, polypropylene, polystyrene, and the like), protein, paper, carbohydrate, lipid monolayer or supported lipid bilayer, and other solid supports. Other materials that may be employed include ceramics, metals, metalloids, semiconductive materials, cements and the like.

In some embodiments, the fusion proteins are organized as an array. The term "array," as used herein, refers to an ordered arrangement of immobilized fusion proteins, in which particular different fusion proteins (i.e., having different PDZ domains) are located at different predetermined sites on the substrate. Because the location of particular fusion proteins on the array is known, binding at that location can be correlated with binding to the PDZ domain situated at that location. Immobilization of fusion proteins on beads (individually or in groups) is another particularly useful approach. In one embodiment, individual fusion proteins are immobilized on beads. In one embodiment, mixtures of distinguishable beads are used. Distinguishable beads are beads that can be separated from each other on the basis of a property such as size, magnetic property, color (e.g., using FACS) or affinity tag (e.g., a bead coated with protein A can be separated from a bead not coated with protein A by using IgG affinity methods). Binding to particular PDZ domain may be determined; similarly, the effect of test compounds (i.e., agonists and antagonists of binding) may be determined.

Methods for immobilizing proteins are known, and include covalent and non-covalent methods. One suitable immobilization method is antibody-mediated immobilization. According to this method, an antibody specific for the sequence of an "immobilization domain" of the PDZ-domain containing protein is itself immobilized on the substrate (e.g., by adsorption). One advantage of this approach is that a single antibody may be adhered to the substrate and used for immobilization of a number of polypeptides (sharing the same immobilization domain). For example, an immobilization domain consisting of poly-histidine (Bush et al, 1991, *J. Biol Chem* 266:13811-14) can be bound by an anti-histidine monoclonal antibody (R&D Systems,

Minneapolis, MN); an immobilization domain consisting of secreted alkaline phosphatase ("SEAP") (Berger et al, 1988, *Gene* 66:1-10) can be bound by anti-SEAP (Sigma Chemical Company, St. Louis, MO); an immobilization domain consisting of a FLAG epitope can be bound by anti-FLAG. Other ligand-antiligand immobilization methods are also suitable (e.g., an immobilization domain consisting of protein A sequences (Harlow and Lane, 1988, Antibodies A Laboratory Manual, Cold Spring Harbor Laboratory; Sigma Chemical Co., St. Louis, MO) can be bound by IgG; and an immobilization domain consisting of streptavidin can be bound by biotin (Harlow & Lane, *supra*; Sigma Chemical Co., St. Louis, MO). In a preferred embodiment, the immobilization domain is a GST moiety, as described herein.

When antibody-mediated immobilization methods are used, glass and plastic are especially useful substrates. The substrates may be printed with a hydrophobic (e.g., Teflon) mask to form wells. Preprinted glass slides with 3, 10 and 21 wells per 14.5 cm^2 slide "working area" are available from, e.g., SPI Supplies, West Chester, PA; also see U.S. Patent No. 4,011,350). In certain applications, a large format (12.4 cm x 8.3 cm) glass slide is printed in a 96 well format is used; this format facilitates the use of automated liquid handling equipment and utilization of 96 well format plate readers of various types (fluorescent, colorimetric, scintillation). However, higher densities may be used (e.g., more than 10 or 100 polypeptides per cm^2). See, e.g., MacBeath et al, 2000, *Science* 289:1760-63.

Typically, antibodies are bound to substrates (e.g., glass substrates) by adsorption. Suitable adsorption conditions are well known in the art and include incubation of 0.5-50 μg/ml (e.g., 10 μg/ml) mAb in buffer (e.g., PBS, or 50 to 300 mM Tris, MOPS, HEPES, PIPES, acetate buffers, pHs 6.5 to 8, at 4°C) to 37°C and from 1 hr to more than 24 hours.

Proteins may be covalently bound or noncovalently attached through nonspecific bonding. If covalent bonding between the fusion protein and the surface is desired, the surface will usually be polyfunctional or be capable of being polyfunctionalized. Functional groups which may be present on the surface and used for linking can include carboxylic acids, aldehydes, amino groups, cyano groups, ethylenic groups, hydroxyl groups, mercapto groups and the like. The manner of linking a wide variety of compounds to various surfaces is well known and is amply illustrated in the literature.

F. Results of PDZ-PL Interaction Assays

TABLE 6 shows the predicted results of assays in which specific binding will be detected between TRPM7 and PDZ proteins using the G3 assay described hereinTABLE 8 shows PDZ domains demonstrated to interact with Tat-TRPM7 peptide. **TABLE 7** shows PDZs that have been demonstrated to interact with PLs involved in excitotoxicity.

Table 6 – **PDZs predicted to interact with TRPM7**

TRPM7	NNOS, LIM, KIAA1095, HEMBA1003117, AIPC, KIAA1526, DVL1, DVL2, DVL3, PTPL1, ZO-1, ZO-2, ZO-3, KIAA1719, Mupp1, INADL, Shank 3, MINT1, MINT2, MAGI1, MAGI2, MAGI3, NeDLG, syntenin, PSD-95, hDLG, PAR3, MAST1, MAST2, AF6, SIP1, LIM mystique, KIAA0751, HTRA1, HTRA2, TIP-1, KIAA0316, PICK1,

Table 7 – **PDZs demonstrated to interact with specific PL proteins**

PL	**PDZs that interact**
NMDA Receptor 2s	PSD95, DLG1, DLG2, NeDLG, KIAA0973, Outermembrane Protein, syntrophin alpha 1, TIP1, TIP2, MAGI1, MAGI2, syntrophin beta 1, syntrophin gamma1, LIM-RIL, KIAA1634, KIAA0807
NMDA Receptor 1s	NeDLG, DLG1, PTPL1, PSD-95, MAGI3,
AMPA Rs	
PLC beta, gamma	EBP50,
NNOS internal	

Table 8 - **PDZ domains demonstrated to interact with Tat-TRPM7 peptide**

PDZ Protein	Domain	GenBank #	Nervous System/ Brain Expression	Reference

RIM-2	1	12734165	yes	Mukasa et al. (2004) Brain Pathol. 14:34-42
Mint 1	1 and 2	2625024	yes	Ho et al. (2003) Proc. Natl. Acad. Sci. U.S.A. 100:1409-1414
INADL	3	2370148	yes	Philipp et al. (1997) FEBS Lett. 413:243-248
Syntrophin 1 alpha	1	1145727	yes	Connors et al. (2004) J. Biol. Chem. 279:28387-28392 g45700023 g34455039
SITAC-18	2	8886071	yes	Borrell-Pagès et al. (2000) Mol. Biol. Cell 11:4217-4225
LIM mystique	1	12734250	no	
ZO-1	2	292937	yes	Poliak et al. (2002) J. Cell Biol. 159:361-372
PAR3L	3	18568347	yes	Gao et al. (2002) Gene 294:99-107
MAST2	1	3882334	yes	g47035747
PAR3	3	8037914	yes	Poliak et al. (2002) J. Cell Biol. 159:361-372
NSP [novel serine protease]	1	1621243	yes	g46953413

TABLE 9 - **results of titrations of the Tat-TRPM7 peptide**

PDZ	EC50, uM	error of fit , EC50, uM	ODmax (450 nm)
RIM2 (177.4)	0.047	0.005	2.10
Mint 1 (d1,d2) (36.5a)	0.180	0.016	1.90
TIP1 d1 (54.10)	< 1	ND	1.82
Mint1 d1 (146.5)	*ND*	*ND*	0.33
Mint1 d2 (147.2)	< 2 uM	ND	0.76
INADL d3 (96.3)	0.120	0.015	1.90
MUPP1 d3 (108.3)	0.290	0.037	2.06
Syntrophin 1 alpha d1 (52.5)	0.160	0.017	2.05
SITAC-18 d1 (122.2)	< 1 uM	ND	1.70
SITAC-18 d2(123.2)	0.090	0.013	1.95
LIM Mystique d1 (232.1)	0.090	0.015	2.10
ZO-1 d2 (241.3)	0.033	0.003	2.20
PAR3L d3 (406.1)	0.100	0.017	1.97
MAST2 d1 (174.6)	0.110	0.008	2.10
PAR3 d3 (278.1)	0.018	0.002	2.50
KIAA1284 d1 (191.2)	> 2 uM	ND	0.63

ZO-1 is a PDZ protein that is known to associate with cell surface proteins, junctions and cytoskeletal proteins. TRPC4 has also been shown to colocalize with ZO-1 (Song, X. et. Al. Glia. 2004, Nov 11 [Epub] PMID 15540229). We have demonstrated that TRPM7 interacts with the second PDZ domain of ZO-1. Without being bound by mechanism, we propose that interruption of TRPM7/ZO-1 d2 interaction may reduce the efficiency Ca^{2+} pumping activity of TRPM7/TRP during oxygen/glucose deprivation or ischemia, resulting in protection from ischemia, especially neuroprotection.

MINT1 is a PDZ protein containing 2 PDZ domains and a PTB domain. MINT1 has been shown to associate with KIR2, an inward rectifying potassium channel (Leonoudakis D. et.al. J Biol. Chem. 2004 279(21):22331-46). MINT1 has also been demonstrated to affect transport of NMDA Receptor 2B to the post-synaptic density of neurons (Scorza et al. Epilepsy Res. 2003 57(1): 49-57), and to play a role in transient global ischema in the mouse hippocampus (Nishimura, H. J. Cereb. Blood Flow Metab. 2000 20(10): 1437-45). We have disclosed herein that TRPM7 binds to MINT1. Without being bound by specific mechanism, we propose that disruption of this interaction with a peptide therapeutic or small molecule will reduce excitotoxicity and result in neuroprotection from ischemic damage.

Partitioning-defective homolog 3 (PAR3) is a PDZ protein with three PDZ domains. It is known to localize to tight junctions form a complex that regulates signaling through cdc42/Rac and Protein Kinases C and A (Lin,D. Nat. Cell Biol. 2 (8), 540-547 (2000)). We have disclosed that TRPM7 interacts with PAR3. Without being bound by specific mechanism, we propose that disruption of this interaction with a peptide therapeutic or small molecule will reduce excitotoxicity and result in neuroprotection from ischemic damage.

RIM2 is a PDZ protein known to be involved with secretory vesicle exocytosis and vesicle transport (Fukuda, M. Genes Cells. 2004 Sep;9(9):831-42). We have disclosed that

TRPM7 interacts with RIM2. Without being bound by specific mechanism, we propose that disruption of this interaction with a peptide therapeutic or small molecule will reduce the ability of TRPM7 to function appropriately at the membrane and result in neuroprotection from ischemic damage.

INADL is a PDZ protein with multiple PDZ domains. It is known to organize proteins at the cell membrane photoreceptor complex, including the *Drosophila* TRP channel. We disclose herein that TRPM7 binds the third PDZ domain of INADL. Without being bound by specific mechanism, we propose that disruption of this interaction with a peptide therapeutic or small molecule will reduce the ability of TRPM7 to associate with necessary accessory proteins and thereby function, resulting in neuroprotection from ischemic damage.

G. Measurement of PDZ-Ligand Binding Affinity

The A and G assays of the invention can be used to determine the "apparent affinity" of binding of a PDZ ligand peptide to a PDZ-domain polypeptide. Apparent affinity is determined based on the concentration of one molecule required to saturate the binding of a second molecule (e.g., the binding of a ligand to a receptor). Two particularly useful approaches for quantitation of apparent affinity of PDZ-ligand binding are provided *infra*.

Approach 1:

(1) A GST/PDZ fusion protein, as well as GST alone as a negative control, are bound to a surface (e.g., a 96-well plate) and the surface blocked and washed as described supra for the G assay.

(2) 50 µl per well of a solution of biotinylated PL peptide (e.g. as shown in **TABLE 2**) is added to the surface in increasing concentrations in PBS/BSA (e.g. at 0.1 µM, 0.33 µM, 1 µM, 3.3 µM, 10 µM, 33 µM, and 100 µM). In one embodiment, the PL peptide is allowed to react with the bound GST/PDZ fusion protein (as well as the GST alone negative control) for 10 minutes at 4°C followed by 20 minutes at 25°C. The plate is washed 3 times with ice cold PBS to remove unbound labeled peptide.

(3) The binding of the PL peptide to the immobilized PDZ-domain polypeptide is detected as described supra for the G assay.

(4) For each concentration of peptide, the net binding signal is determined by subtracting the binding of the peptide to GST alone from the binding of the peptide to the GST/PDZ fusion protein. The net binding signal is then plotted as a function of ligand concentration and the plot is fit (e.g. by using the Kaleidagraph Synergy Software, Reading, PA) software package curve fitting algorithm) to the following equation, where "$Signal_{[ligand]}$" is the net binding signal at PL peptide concentration "[ligand]," "K_d" is the apparent affinity of the binding event, and "Saturation Binding" is a constant determined by the curve fitting algorithm to optimize the fit to the experimental data:

$$Signal_{[ligand]} = \text{Saturation Binding} \times ([ligand] / ([ligand] + K_d))$$

For reliable application of the above equation it is necessary that the highest peptide ligand concentration successfully tested experimentally be greater than, or at least similar to, the calculated K_d (equivalently, the maximum observed binding should be similar to the calculated saturation binding). In cases where satisfying the above criteria proves difficult, an alternative approach (infra) can be used.

Approach 2:

(1) A fixed concentration of a PDZ-domain polypeptide and increasing concentrations of a labeled PL peptide (labeled with, for example, biotin or fluorescein, see **TABLE 2** and **TABLE 4** for representative peptide amino acid sequences) are mixed together in solution and allowed to react. In one embodiment, preferred peptide concentrations are 0.1 μM, 1 μM, 10 μM, 100 μM, 1 mM. In various embodiments, appropriate reaction times can range from 10 minutes to 2 days at temperatures ranging from 4°C to 37°C. In some embodiments, the identical reaction can also be carried out using a non-PDZ domain-containing protein as a control (e.g., if the PDZ-domain polypeptide is fusion protein, the fusion partner can be used).

(2) PDZ-ligand complexes can be separated from unbound labeled peptide using a variety of methods known in the art. For example, the complexes can be separated using high performance size-exclusion chromatography (HPSEC, gel filtration) (Rabinowitz et al., 1998,

Immunity 9:699), affinity chromatography (e.g. using glutathione SEPHAROSE beads), and affinity absorption (e.g., by binding to an anti-GST-coated plate as described *supra*).

(3) The PDZ-ligand complex is detected based on presence of the label on the peptide ligand using a variety of methods and detectors known to one of skill in the art. For example, if the label is fluorescein and the separation is achieved using HPSEC, an in-line fluorescence detector can be used. The binding can also be detected as described *supra* for the G assay.

(4) The PDZ-ligand binding signal is plotted as a function of ligand concentration and the plot is fit. (e.g., by using the Kaleidagraph software package curve fitting algorithm) to the following equation, where "$Signal_{[ligand]}$" is the binding signal at PL peptide concentration "[ligand]," "K_d" is the apparent affinity of the binding event, and "Saturation Binding" is a constant determined by the curve fitting algorithm to optimize the fit to the experimental data:

$$Signal_{[Ligand]} = \text{Saturation Binding} \times ([\text{ligand}] / ([\text{ligand} + K_d]))$$

Measurement of the affinity of a labeled peptide ligand binding to a PDZ-domain polypeptide is useful because knowledge of the affinity (or apparent affinity) of this interaction allows rational design of inhibitors of the interaction with known potency. The potency of inhibitors in inhibition would be similar to (i.e. within one-order of magnitude of) the apparent affinity of the labeled peptide ligand binding to the PDZ-domain.

Thus, in one aspect, the invention provides a method of determining the apparent affinity of binding between a PDZ domain and a ligand by immobilizing a polypeptide comprising the PDZ domain and a non-PDZ domain on a surface, contacting the immobilized polypeptide with a plurality of different concentrations of the ligand, determining the amount of binding of the ligand to the immobilized polypeptide at each of the concentrations of ligand, and calculating the apparent affinity of the binding based on that data. Typically, the polypeptide comprising the PDZ domain and a non-PDZ domain is a fusion protein. In one embodiment, the e.g., fusion protein is GST-PDZ fusion protein, but other polypeptides can also be used (e.g., a fusion protein including a PDZ domain and any of a variety of epitope tags, biotinylation signals and the like) so long as the polypeptide can be immobilized in an orientation that does not abolish the ligand binding properties of the PDZ domain, e.g, by tethering the polypeptide to the surface via the non-PDZ domain via an anti-domain antibody and leaving the PDZ domain as the

free end. It was discovered, for example, reacting a PDZ-GST fusion polypeptide directly to a plastic plate provided suboptimal results. The calculation of binding affinity itself can be determined using any suitable equation (e.g., as shown *supra*; also see Cantor and Schimmel (1980) Biophysical Chemistry WH Freeman & Co., San Francisco) or software.

Thus, in a preferred embodiment, the polypeptide is immobilized by binding the polypeptide to an immobilized immunoglobulin that binds the non-PDZ domain (e.g., an anti-GST antibody when a GST-PDZ fusion polypeptide is used). In a preferred embodiment, the step of contacting the ligand and PDZ-domain polypeptide is carried out under the conditions provided *supra* in the description of the G assay. It will be appreciated that binding assays are conveniently carried out in multiwell plates (e.g., 24-well, 96-well plates, or 384 well plates).

The present method has considerable advantages over other methods for measuring binding affinities PDZ-PL affinities, which typically involve contacting varying concentrations of a GST-PDZ fusion protein to a ligand–coated surface. For example, some previously described methods for determining affinity (e.g., using immobilized ligand and GST-PDZ protein in solution) did not account for oligomerization state of the fusion proteins used, resulting in potential errors of more than an order of magnitude.

Although not sufficient for quantitative measurement of PDZ-PL binding affinity, an estimate of the relative strength of binding of different PDZ-PL pairs can be made based on the absolute magnitude of the signals observed in the G assay. This estimate will reflect several factors, including biologically relevant aspects of the interaction, including the affinity and the dissociation rate. For comparisons of different ligands binding to a given PDZ domain-containing protein, differences in absolute binding signal likely relate primarily to the affinity and/or dissociation rate of the interactions of interest.

H. Assays to Identify Novel PDZ Domain Binding Moieties and to Identify Modulators of PDZ Protein-PL Protein Binding

Although described *supra* primarily in terms of identifying interactions between PDZ-domain polypeptides and PL proteins, the assays described *supra* and other assays can also be used to identify the binding of other molecules (e.g., peptide mimetics, small molecules, and the like) to PDZ domain sequences. For example, using the assays disclosed herein, combinatorial and other libraries of compounds can be screened, e.g., for molecules that

specifically bind to PDZ domains. Screening of libraries can be accomplished by any of a variety of commonly known methods. See, e.g., the following references, which disclose screening of peptide libraries: Parmley and Smith, 1989, Adv. Exp. Med. Biol. 251:215-218; Scott and Smith, 1990, Science 249:386-390; Fowlkes et al., 1992; BioTechniques 13:422-427; Oldenburg et al., 1992, Proc. Natl. Acad. Sci. USA 89:5393-5397; Yu et al., 1994, Cell 76:933-945; Staudt et al., 1988, Science 241:577-580; Bock et al., 1992, Nature 355:564-566; Tuerk et al., 1992, Proc. Natl. Acad. Sci. USA 89:6988-6992; Ellington et al., 1992, Nature 355:850-852; U.S. Patent No. 5,096,815, U.S. Patent No. 5,223,409, and U.S. Patent No. 5,198,346, all to Ladner et al.; Rebar and Pabo, 1993, Science 263:671-673; and PCT Publication No. WO 94/18318.

In a specific embodiment, screening can be carried out by contacting the library members with a PDZ-domain polypeptide immobilized on a solid support (e.g. as described *supra* in the G assay) and harvesting those library members that bind to the protein. Examples of such screening methods, termed "panning" techniques are described by way of example in Parmley and Smith, 1988, Gene 73:305-318; Fowlkes et al., 1992, BioTechniques 13:422-427; PCT Publication No. WO 94/18318; and in references cited hereinabove.

In another embodiment, the two-hybrid system for selecting interacting proteins in yeast (Fields and Song, 1989, Nature 340:245-246; Chien et al., 1991, Proc. Natl. Acad. Sci. USA 88:9578-9582) can be used to identify molecules that specifically bind to a PDZ domain-containing protein. Furthermore, the identified molecules are further tested for their ability to inhibit transmembrane receptor interactions with a PDZ domain.

In one aspect of the invention, antagonists of an interaction between a PDZ protein and a PL protein are identified. In one embodiment, a modification of the A assay described *supra* is used to identify antagonists. In one embodiment, a modification of the G assay described *supra* is used to identify antagonists.

In certain embodiments, screening assays are used to detect molecules that specifically bind to PDZ domains. Such molecules are useful as agonists or antagonists of PDZ-protein-mediated cell function (e.g., cell activation, e.g., T cell activation, vesicle transport, cytokine release, growth factors, transcriptional changes, cytoskeleton rearrangement, cell movement, chemotaxis, intercellular signaling, regulation of synaptic function, neuronal excitation, cytoskeletal integrity, and neurotransmitter release). In one embodiment, such assays

are performed to screen for leukocyte activation inhibitors for drug development. The invention thus provides assays to detect molecules that specifically bind to PDZ domain-containing proteins. For example, recombinant cells expressing PDZ domain-encoding nucleic acids can be used to produce PDZ domains in these assays and to screen for molecules that bind to the domains. Molecules are contacted with the PDZ domain (or fragment thereof) under conditions conducive to binding, and then molecules that specifically bind to such domains are identified. Methods that can be used to carry out the foregoing are commonly known in the art.

It will be appreciated by the ordinarily skilled practitioner that, in one embodiment, antagonists are identified by conducting the A or G assays in the presence and absence of a known or candidate antagonist. When decreased binding is observed in the presence of a compound, that compound is identified as an antagonist. Increased binding in the presence of a compound signifies that the compound is an agonist.

For example, in one assay, a test compound can be identified as an inhibitor (antagonist) of binding between a PDZ protein and a PL protein by contacting a PDZ domain polypeptide and a PL peptide or protein in the presence and absence of the test compound, under conditions in which they would (but for the presence of the test compound) form a complex, and detecting the formation of the complex in the presence and absence of the test compound. It will be appreciated that less complex formation in the presence of the test compound than in the absence of the compound indicates that the test compound is an inhibitor of a PDZ protein -PL protein binding.

In one embodiment, the G assay is used in the presence or absence of an candidate inhibitor. In one embodiment, the A assay is used in the presence or absence of a candidate inhibitor.

In one embodiment (in which a G assay is used), one or more PDZ domain-containing GST-fusion proteins are bound to the surface of wells of a 96-well plate as described *supra* (with appropriate controls including nonfusion GST protein). All fusion proteins are bound in multiple wells so that appropriate controls and statistical analysis can be done. A test compound in BSA/PBS (typically at multiple different concentrations) is added to wells. Immediately thereafter, 30 µl of a detectably labeled (e.g., biotinylated) PL peptide or protein known to bind to the relevant PDZ domain (see, e.g., **TABLE 5**) is added in each of the wells at a final concentration of, e.g., between about 2 µM and about 40 µM, typically 5 µM, 15 µM, or

25 μM. This mixture is then allowed to react with the PDZ fusion protein bound to the surface for 10 minutes at 4°C followed by 20 minutes at 25°C. The surface is washed free of unbound PL polypeptide three times with ice cold PBS and the amount of binding of the polypeptide in the presence and absence of the test compound is determined. Usually, the level of binding is measured for each set of replica wells (e.g. duplicates) by subtracting the mean GST alone background from the mean of the raw measurement of polypeptide binding in these wells.

In an alternative embodiment, the A assay is carried out in the presence or absence of a test candidate to identify inhibitors of PL-PDZ interactions.

If assays are conducted in the presence of test compound and compared against binding in the absence of test compound, then the assay can be conducted to determine if the difference between binding in the presence and absence of the test compound is a statistically significant difference.

In certain screening assays, assays are conducted to identify compounds that can inhibit a binding interaction between a TRP channel or TRP associated protein and a PDZ listed in **TABLE 5**. In other screening assays involve screening to identify an inhibitor that interferes with binding between TRPM7 and a PDZ listed in **TABLE 5**.

In one embodiment, a test compound is determined to be a specific inhibitor of the binding of the PDZ domain (P) and a PL (L) sequence when, at a test compound concentration of less than or equal to 1 mM (e.g., less than or equal to: 500 μM, 100 μM, 10 μM, 1 μM, 100 nM or 1 nM) the binding of P to L in the presence of the test compound less than about 50% of the binding in the absence of the test compound (in various embodiments, less than about 25%, less than about 10%, or less than about 1%). Preferably, the net signal of binding of P to L in the presence of the test compound plus six (6) times the standard error of the signal in the presence of the test compound is less than the binding signal in the absence of the test compound.

In one embodiment, assays for an inhibitor are carried out using a single PDZ protein-PL protein pair (e.g., a PDZ domain fusion protein and a PL peptide or protein). In a related embodiment, the assays are carried out using a plurality of pairs, such as a plurality of different pairs listed in **TABLES 2, 4** and **5**.

In some embodiments, it is desirable to identify compounds that, at a given concentration, inhibit the binding of one PL-PDZ pair, but do not inhibit (or inhibit to a lesser

degree) the binding of a specified second PL-PDZ pair. These antagonists can be identified by carrying out a series of assays using a candidate inhibitor and different PL-PDZ pairs (e.g., as shown in **TABLE 6, 7, and 8**) and comparing the results of the assays. All such pairwise combinations are contemplated by the invention (e.g., test compound inhibits binding of PL_1 to PDZ_1 to a greater degree than it inhibits binding of PL_1 to PDZ_2 or PL_2 to PDZ_2). Importantly, it will be appreciated that, based on the data provided in **TABLE 6, 7, and 8** and disclosed herein (and additional data that can be generated using the methods described herein) inhibitors with different specificities can readily be designed.

For example, according to the invention, the K_i ("potency") of an inhibitor of a PDZ-PL interaction can be determined. K_i is a measure of the concentration of an inhibitor required to have a biological effect. For example, administration of an inhibitor of a PDZ-PL interaction in an amount sufficient to result in an intracellular inhibitor concentration of at least between about 1 and about 100 K_i is expected to inhibit the biological response mediated by the target PDZ-PL interaction. In one aspect of the invention, the K_d measurement of PDZ-PL binding as determined using the methods *supra* is used in determining K_i.

Thus, in one aspect, the invention provides a method of determining the potency (K_i) of an inhibitor or suspected inhibitor of binding between a PDZ domain and a ligand by immobilizing a polypeptide comprising the PDZ domain and a non-PDZ domain on a surface, contacting the immobilized polypeptide with a plurality of different mixtures of the ligand and inhibitor, wherein the different mixtures comprise a fixed amount of ligand and different concentrations of the inhibitor, determining the amount of ligand bound at the different concentrations of inhibitor, and calculating the K_i of the binding based on the amount of ligand bound in the presence of different concentrations of the inhibitor. In an embodiment, the polypeptide is immobilized by binding the polypeptide to an immobilized immunoglobulin that binds the non-PDZ domain. This method, which is based on the G assay described *supra*, is particularly suited for high-throughput analysis of the K_i for inhibitors of PDZ-ligand interactions. Further, using this method, the inhibition of the PDZ-ligand interaction itself is measured, without distortion of measurements by avidity effects.

Typically, at least a portion of the ligand is detectably labeled to permit easy quantitation of ligand binding.

It will be appreciated that the concentration of ligand and concentrations of inhibitor are selected to allow meaningful detection of inhibition. Thus, the concentration of the ligand whose binding is to be blocked is close to or less than its binding affinity (e.g., preferably less than the 5x K_d of the interaction, more preferably less than 2x K_d, most preferably less than 1x K_d). Thus, the ligand is typically present at a concentration of less than 2 K_d (e.g., between about 0.01 K_d and about 2 K_d) and the concentrations of the test inhibitor typically range from 1 nM to 100 μM (e.g. a 4-fold dilution series with highest concentration 10 μM or 1 mM). In a preferred embodiment, the K_d is determined using the assay disclosed *supra.*

The K_i of the binding can be calculated by any of a variety of methods routinely used in the art, based on the amount of ligand bound in the presence of different concentrations of the inhibitor. In an illustrative embodiment, for example, a plot of labeled ligand binding versus inhibitor concentration is fit to the equation:

$$S_{inhibitor} = S_0 * K_i / ([I] + K_i)$$

where $S_{inhibitor}$ is the signal of labeled ligand binding to immobilized PDZ domain in the presence of inhibitor at concentration [I] and S_0 is the signal in the absence of inhibitor (i.e., [I] = 0). Typically [I] is expressed as a molar concentration.

In another aspect of the invention, an enhancer (sometimes referred to as, augmentor or agonist) of binding between a PDZ domain and a ligand is identified by immobilizing a polypeptide comprising the PDZ domain and a non-PDZ domain on a surface, contacting the immobilized polypeptide with the ligand in the presence of a test agent and determining the amount of ligand bound, and comparing the amount of ligand bound in the presence of the test agent with the amount of ligand bound by the polypeptide in the absence of the test agent. At least two-fold (often at least 5-fold) greater binding in the presence of the test agent compared to the absence of the test agent indicates that the test agent is an agent that enhances the binding of the PDZ domain to the ligand. As noted *supra*, agents that enhance PDZ-ligand interactions are useful for disruption (dysregulation) of biological events requiring normal PDZ-ligand function (e.g., cancer cell division and metastasis, and activation and migration of immune cells, intercellular communication, neurotransmitter release, membrane receptor turnover, second messenger signaling responsible for cell homeostasis and function).

The invention also provides methods for determining the "potency" or "$K_{enhancer}$" of an enhancer of a PDZ- ligand interaction. For example, according to the invention, the

$K_{enhancer}$ of an enhancer of a PDZ-PL interaction can be determined, e.g., using the Kd of PDZ-PL binding as determined using the methods described *supra*. $K_{enhancer}$ is a measure of the concentration of an enhancer expected to have a biological effect. For example, administration of an enhancer of a PDZ-PL interaction in an amount sufficient to result in an intracellular inhibitor concentration of at least between about 0.1 and about 100 $K_{enhancer}$ (e.g., between about 0.5 and about 50 $K_{enhancer}$) is expected to disrupt the biological response mediated by the target PDZ-PL interaction.

Thus, in one aspect the invention provides a method of determining the potency ($K_{enhancer}$) of an enhancer or suspected enhancer of binding between a PDZ domain and a ligand by immobilizing a polypeptide comprising the PDZ domain and a non-PDZ domain on a surface, contacting the immobilized polypeptide with a plurality of different mixtures of the ligand and enhancer, wherein the different mixtures comprise a fixed amount of ligand, at least a portion of which is detectably labeled, and different concentrations of the enhancer, determining the amount of ligand bound at the different concentrations of enhancer, and calculating the potency ($K_{enhancer}$) of the enhancer from the binding based on the amount of ligand bound in the presence of different concentrations of the enhancer. Typically, at least a portion of the ligand is detectably labeled to permit easy quantitation of ligand binding. This method, which is based on the G assay described *supra,* is particularly suited for high-throughput analysis of the $K_{enhancer}$ for enhancers of PDZ-ligand interactions.

It will be appreciated that the concentration of ligand and concentrations of enhancer are selected to allow meaningful detection of enhanced binding. Thus, the ligand is typically present at a concentration of between about 0.01 K_d and about 0.5 K_d and the concentrations of the test agent/enhancer typically range from 1 nM to 1 mM (e.g. a 4-fold dilution series with highest concentration 10 μM or 1 mM). In a preferred embodiment, the K_d is determined using the assay disclosed *supra*.

The potency of the binding can be determined by a variety of standard methods based on the amount of ligand bound in the presence of different concentrations of the enhancer or augmentor. For example, a plot of labeled ligand binding versus enhancer concentration can be fit to the equation:

$$S([E]) = S(0) + (S(0)*(D_{enhancer}-1)*[E]/([E]+ K_{enhancer})$$

where "$K_{enhancer}$" is the potency of the augmenting compound, and "$D_{enhancer}$" is the fold-increase in binding of the labeled ligand obtained with addition of saturating amounts of the enhancing compound, [E] is the concentration of the enhancer. It will be understood that saturating amounts are the amount of enhancer such that further addition does not significantly increase the binding signal. Knowledge of "$K_{enhancer}$" is useful because it describes a concentration of the augmenting compound in a target cell that will result in a biological effect due to dysregulation of the PDZ-PL interaction. Typical therapeutic concentrations are between about 0.1 and about 100 $K_{enhancer}$.

V. Validation of Binding Assays

Compounds identified in the foregoing binding assays can be further analyzed using a variety of biological assays to confirm that the ability of the compound to inhibit a PDZ:PL protein interaction actually inhibits a cellular activity correlated with the PDZ:PL binding interaction. Alternatively, these assays can be used directly to assay the activity of a potential inhibitory compound without conducting a binding assay beforehand. These assays can be conducted using various in vitro assays, or in vivo assays using various appropriate animal model systems.

The PDZ:PL binding interactions described herein include those involved in various biological activities in neurons. As already noted, one set of cellular activities of interest are those associated with various types of neurological disorders or injury, such as cellular responses associated with stroke and ischemia. Because neurological injury is often associated with cell death, apoptosis and excitotoxicity responses, assays for each of these responses can be conducted to validate the inhibitory activity of a compound identified through a binding assay.

For example, a variety of different parameters can be monitored to assess toxicity. Examples of such parameters include, but are not limited to, cell proliferation, monitoring activation of cellular pathways for toxicological responses by gene or protein expression analysis, DNA fragmentation, changes in the composition of cellular membranes, membrane permeability, activation of components of death-receptors or downstream signaling pathways (e.g., caspases), generic stress responses, NF-kappaB activation and responses to mitogens. Related assays are used to assay for apoptosis (a programmed process of cell death) and necrosis, including cGMP formation and NO formation. The following are illustrative of the type of

biological assays that can be conducted to assess whether a inhibitory agent has a protective effect against neuronal injury or disease.

A. Morphological Changes

Apoptosis in many cell types is correlated with altered morphological appearances. Examples of such alterations include, but are not limited to, plasma membrane blebbing, cell shape change, loss of substrate adhesion properties. Such changes are readily detectable with a light microscope. Cells undergoing apoptosis can also be detected by fragmentation and disintegration of chromosomes. These changes can be detected using light microscopy and/or DNA or chromatin specific dyes.

B. Altered Membrane Permeability

Often the membranes of cells undergoing apoptosis become increasingly permeable. This change in membrane properties can be readily detected using vital dyes (e.g., propidium iodide and trypan blue). Dyes can be used to detect the presence of necrotic cells. For example, certain methods utilize a green-fluorescent LIVE/DEAD Cytotoxicity Kit #2, available from Molecular Probes. The dye specifically reacts with cellular amine groups. In necrotic cells, the entire free amine content is available to react with the dye, thus resulting in intense fluorescent staining. In contrast, only the cell-surface amines of viable cells are available to react with the dye. Hence, the fluorescence intensity for viable cells is reduced significantly relative to necrotic cells (see, e.g., Haugland, 1996 *Handbook of Fluorescent Probes and Research Chemicals*, 6th ed., Molecular Probes, OR).

C. Dysfunction of Mitochondrial Membrane Potential

Mitochondria provide direct and indirect biochemical regulation of diverse cellular processes as the main energy source in cells of higher organisms. These processes include the electron transport chain activity, which drives oxidative phosphorylation to produce metabolic energy in the form of adenosine triphosphate (i.e., ATP). Altered or defective mitochondrial activity can result in mitochondrial collapse called the "permeability transition" or mitochondrial permeability transition. Proper mitochondrial functioning requires maintenance of

the membrane potential established across the membrane. Dissipation of the membrane potential prevents ATP synthesis and thus halts or restricts the production of a vital biochemical energy source.

Consequently, a variety of assays designed to assess toxicity and cell death involve monitoring the effect of a test agent on mitochondrial membrane potentials or on the mitochondrial permeability transition. One approach is to utilize fluorescent indicators (see, e.g., Haugland, 1996 *Handbook of Fluorescent Probes and Research Chemicals*, 6th ed., Molecular Probes, OR, pp. 266-274 and 589-594). Various non-fluorescent probes can also be utilized (see, e.g., Kamo et al. (1979) J. Membrane Biol. 49:105). Mitochondrial membrane potentials can also be determined indirectly from mitochondrial membrane permeability (see, e.g., Quinn (1976) *The Molecular Biology of Cell Membranes*, University Park Press, Baltimore, Maryland, pp. 200-217). Further guidance on methods for conducting such assays is provided in PCT publication WO 00/19200 to Dykens et al.

D. Caspase Activation

Apoptosis is the process of programmed cell death and involves the activation of a genetic program when cells are no longer needed or have become seriously damaged. Apoptosis involves a cascade of biochemical events and is under the regulation of a number of different genes. One group of genes act as effectors of apoptosis and are referred to as the interleukin-1 beta.converting enzyme (ICE) family of genes. These genes encode a family of cysteine proteases whose activity is increased in apoptosis. The ICE family of proteases is generically referred to as caspase enzymes. The "c" in the name reflects the fact that the enzymes are cysteine proteases, while "aspase" refers to the ability of these enzymes to cleave after aspartic acid residues.

Consequently, some assays for apoptosis are based upon the observation that caspases are induced during apoptosis. Induction of these enzymes can be detected by monitoring the cleavage of specifically-recognized substrates for these enzymes. A number of naturally occurring and synthetic protein substrates are known (see, e.g., Ellerby et al. (1997) J. Neurosci. 17:6165; Kluck, et al. (1997) Science 275:1132; Nicholson et al. (1995) Nature 376:37; and Rosen and Casciola-Rosen (1997) J. Cell Biochem. 64:50). Methods for preparing a number of different substrates that can be utilized in these assays are described in U.S. Patent

No. 5,976,822. This patent also describes assays that can be conducted using whole cells that are amendable to certain of the microfluidic devices described herein. Other methods using FRET techniques are discussed in Mahajan, et al. (1999) Chem. Biol. 6:401-9; and Xu, et al. (1998) Nucl. Acids. Res. 26:2034-5.

E. Cytochrome c Release

In healthy cells, the inner mitochondrial membrane is impermeable to macromolecules. Thus, one indicator of cell apoptosis is the release or leakage of cytochrome c from the mitochondria. Detection of cytochrome c can be performed using spectroscopic methods because of the inherent absorption properties of the protein. Thus, one detection option with the present devices is to place the cells within a holding space and monitor absorbance at a characteristic absorption wavelength for cytochrome c. Alternatively, the protein can be detected using standard immunological methods (e.g., ELISA assays) with an antibody that specifically binds to cytochrome c (see, e.g., Liu et al. (1996) Cell 86:147).

F. Assays for Cell Lysis

The final stage of cell death is typically lysis of the cell. When cells die they typically release a mixture of chemicals, including nucleotides, and a variety of other substances (e.g., proteins and carbohydrates) into their surroundings. Some of the substances released include ADP and ATP, as well as the enzyme adenylate cyclase, which catalyzes the conversion of ADP to ATP in the presence of excess ADP. Thus, certain assays involve providing sufficient ADP in the assay medium to drive the equilibrium towards the generation of ATP which can subsequently be detected via a number of different means. One such approach is to utilize a luciferin/luciferase system that is well known to those of ordinary skill in the art in which the enzyme luciferase utilizes ATP and the substrate luciferin to generate a photometrically detectable signal. Further details regarding certain cell lysis assays that can be performed are set forth in PCT publication WO 00/70082.

G. Ischemic Model Systems

Methods for assaying whether a compound can confer protective neurological effects against ischemia and stroke are discussed by Aarts, et al. (Science 298:846-850, 2002).

In general, this assay involves subjecting rats to a middle cerebral artery occlusion (MCAO) for a relatively short period of time (e.g., about 90 minutes). MCAO can be induced using various methods, including an intraluminal suture method (see, e.g., Longa, E.Z. et al. (1989) Stroke 20:84; and Belayev, L., et al. (1996) Stroke 27:1616). A composition containing the putative inhibitor is introduced into the rat using conventional methods (e.g., via intravenous injection). To evaluate the composition's prophylactic effect, the composition is administered before performing MCAO. If the compound is to be evaluated for its ability to mitigate against an ischemic event that has already occurred, the composition with the compound is introduced after MCAO has been initiated. The extent of cerebral infarction is then evaluated using various measures of neurological function. Examples of such measures include the postural reflex test (Bederson, J.B. et al. (1986) Stroke 17:472) and the forelimb placing test (De Ryck, M. et al. (1989) Stroke 20:1383). Methods are also described in Aarts et al (supra) assessing the effects of NMDA-induced excitotoxicity using in vitro assays.

VI. Global Analysis of PDZ-PL Interactions

As described *supra*, the present invention provides powerful methods for analysis of PDZ-ligand interactions, including high-throughput methods such as the G assay and affinity assays described *supra*. In one embodiment of the invention, the affinity is determined for a particular ligand and a plurality of PDZ proteins. Typically the plurality is at least 5, and often at least 25, or at least 40 different PDZ proteins. In a preferred embodiment, the plurality of different PDZ proteins are from a particular tissue (e.g., central nervous system) or a particular class or type of cell, (e.g., a neuron) and the like. In a most preferred embodiment, the plurality of different PDZ proteins represents a substantial fraction (e.g., typically a majority, more often at least 80%) of all of the PDZ proteins known to be, or suspected of being, expressed in the tissue or cell(s), e.g., all of the PDZ proteins known to be present in neuronal cells. In an embodiment, the plurality is at least 50%, usually at least 80%, at least 90% or all of the PDZ proteins disclosed herein as being expressed in neuronal cells.

In one embodiment of the invention, the binding of a ligand to the plurality of PDZ proteins is determined. Using this method, it is possible to identify a particular PDZ domain bound with particular specificity by the ligand. The binding may be designated as "specific" if the affinity of the ligand to the particular PDZ domain is at least 2-fold that of the

binding to other PDZ domains in the plurality (e.g., present in that cell type). The binding is deemed "very specific" if the affinity is at least 10-fold higher than to any other PDZ in the plurality or, alternatively, at least 10-fold higher than to at least 90%, more often 95% of the other PDZs in a defined plurality. Similarly, the binding is deemed "exceedingly specific" if it is at least 100-fold higher. For example, a ligand could bind to 2 different PDZs with an affinity of 1 μM and to no other PDZs out of a set 40 with an affinity of less than 100 μM. This would constitute specific binding to those 2 PDZs. Similar measures of specificity are used to describe binding of a PDZ to a plurality of PLs.

It will be recognized that high specificity PDZ-PL interactions represent potentially more valuable targets for achieving a desired biological effect. The ability of an inhibitor or enhancer to act with high specificity is often desirable. In particular, the most specific PDZ-ligand interactions are also the best therapeutic targets, allowing specific inhibition of the interaction.

Thus, in one embodiment, the invention provides a method of identifying a high specificity interaction between a particular PDZ domain and a ligand known or suspected of binding at least one PDZ domain, by providing a plurality of different immobilized polypeptides, each of said polypeptides comprising a PDZ domain and a non-PDZ domain; determining the affinity of the ligand for each of said polypeptides, and comparing the affinity of binding of the ligand to each of said polypeptides, wherein an interaction between the ligand and a particular PDZ domain is deemed to have high specificity when the ligand binds an immobilized polypeptide comprising the particular PDZ domain with at least 2-fold higher affinity than to immobilized polypeptides not comprising the particular PDZ domain.

In a related aspect, the affinity of binding of a specific PDZ domain to a plurality of ligands (or suspected ligands) is determined. For example, in one embodiment, the invention provides a method of identifying a high specificity interaction between a PDZ domain and a particular ligand known or suspected of binding at least one PDZ domain, by providing an immobilized polypeptide comprising the PDZ domain and a non-PDZ domain; determining the affinity of each of a plurality of ligands for the polypeptide, and comparing the affinity of binding of each of the ligands to the polypeptide, wherein an interaction between a particular ligand and the PDZ domain is deemed to have high specificity when the ligand binds an immobilized polypeptide comprising the PDZ domain with at least 2-fold higher affinity than

other ligands tested. Thus, the binding may be designated as "specific" if the affinity of the PDZ to the particular PL is at least 2-fold that of the binding to other PLs in the plurality (e.g., present in that cell type). The binding is deemed "very specific" if the affinity is at least 10-fold higher than to any other PL in the plurality or, alternatively, at least 10-fold higher than to at least 90%, more often 95% of the other PLs in a defined plurality. Similarly, the binding is deemed "exceedingly specific" if it is at least 100-fold higher. Typically the plurality is at least 5 different ligands, more often at least 10.

1. Use of Array for Global Predictions

One discovery of the present inventors relates to the important and extensive roles played by interactions between PDZ proteins and PL proteins, particularly in the biological function of neuronal cells. Further, it has been discovered that valuable information can be ascertained by analysis (e.g., simultaneous analysis) of a large number of PDZ-PL interactions. In a preferred embodiment, the analysis encompasses all of the PDZ proteins expressed in a particular tissue (e.g., brain) or type or class of cell (e.g., neuron). Alternatively, the analysis encompasses at least about 5, or at least about 10, or at least about 12, or at least about 15 and often at least 50 different polypeptides, up to about 60, about 80, about 100, about 150, about 200, or even more different polypeptides; or a substantial fraction (e.g., typically a majority, more often at least 80%) of all of the PDZ proteins known to be, or suspected of being, expressed in the tissue or cell(s), (e.g., all of the PDZ proteins known to be present in neurons).

It will be recognized that the arrays and methods of the invention are directed to analyze of PDZ and PL interactions, and involve selection of such proteins for analysis. While the devices and methods of the invention may include or involve a small number of control polypeptides, they typically do not include significant numbers of proteins or fusion proteins that do not include either PDZ or PL domains (e.g., typically, at least about 90% of the arrayed or immobilized polypeptides in a method or device of the invention is a PDZ or PL sequence protein, more often at least about 95%, or at least about 99%).

It will be apparent from this disclosure that analysis of the relatively large number of different interactions preferably takes place simultaneously. In this context, "simultaneously" means that the analysis of several different PDZ-PL interactions (or the effect of a test agent on such interactions) is assessed at the same time. Typically the analysis is carried out in a high

throughput (e.g., robotic) fashion. One advantage of this method of simultaneous analysis is that it permits rigorous comparison of multiple different PDZ-PL interactions. For example, as explained in detail elsewhere herein, simultaneous analysis (and use of the arrays described *infra*) facilitates, for example, the direct comparison of the effect of an agent (e.g., a potential interaction inhibitor) on the interactions between a substantial portion of PDZs and/or PLs in a tissue or cell.

Accordingly, in one aspect, the invention provides an array of immobilized polypeptide comprising the PDZ domain and a non-PDZ domain on a surface. Typically, the array comprises at least about 5, or at least about 10, or at least about 12, or at least about 15 and often at least 50 different polypeptides. In one preferred embodiment, the different PDZ proteins are from a particular tissue (e.g., central nervous system) or a particular class or type of cell, (e.g., a neuron) and the like. In a most preferred embodiment, the plurality of different PDZ proteins represents a substantial fraction (e.g., typically a majority, more often at least 60%, 70% or 80%) of all of the PDZ proteins known to be, or suspected of being, expressed in the tissue or cell(s), (e.g., all of the PDZ proteins known to be present in neurons).

Certain embodiments are arrays which include a plurality, usually at least 5, 10, 25, or 50 PDZ proteins present in a particular cell of interest. In this context, "array" refers to an ordered series of immobilized polypeptides in which the identity of each polypeptide is associated with its location. In some embodiments the plurality of polypeptides are arrayed in a "common" area such that they can be simultaneously exposed to a solution (e.g., containing a ligand or test agent). For example, the plurality of polypeptides can be on a slide, plate or similar surface, which may be plastic, glass, metal, silica, beads or other surface to which proteins can be immobilized. In a different embodiment, the different immobilized polypeptides are situated in separate areas, such as different wells of multi-well plate (e.g., a 24-well plate, a 96-well plate, a 384 well plate, and the like). It will be recognized that a similar advantage can be obtained by using multiple arrays in tandem.

2. Analysis of PDZ-PL Inhibition Profile

In one aspect, the invention provides a method for determining if a test compound inhibits any PDZ-ligand interaction in large set of PDZ-ligand interaction (e.g., a plurality of the PDZ-ligand interactions described in **TABLE 6** and **TABLE 7 and TABLE 8**; a majority of the

PDZ-ligands identified in a particular cell or tissue as described *supra* (e.g., neurons) and the like. In one embodiment, the PDZ domains of interest are expressed as GST-PDZ fusion proteins and immobilized as described herein. For each PDZ domain, a labeled ligand that binds to the domain with a known affinity is identified as described herein.

For any known or suspected modulator (e.g., inhibitor) of a PDL-PL interaction(s), it is useful to know which interactions are inhibited (or augmented). For example, an agent that inhibits all PDZ-PL interactions in a cell (e.g., a neuron) will have different uses than an agent that inhibits only one, or a small number, of specific PDZ-PL interactions. The profile of PDZ interactions inhibited by a particular agent is referred to as the "inhibition profile" for the agent, and is described in detail below. The profile of PDZ interactions enhanced by a particular agent is referred to as the "enhancement profile" for the agent. It will be readily apparent to one of skill guided by the description of the inhibition profile how to determine the enhancement profile for an agent. The present invention provides methods for determining the PDZ interaction (inhibition/enhancement) profile of an agent in a single assay.

In one aspect, the invention provides a method for determining the PDZ-PL inhibition profile of a compound by providing (i) a plurality of different immobilized polypeptides, each of said polypeptides comprising a PDZ domain and a non-PDZ domain and (ii) a plurality of corresponding ligands, wherein each ligand binds at least one PDZ domain in (i), then contacting each of said immobilized polypeptides in (i) with a corresponding ligand in (ii) in the presence and absence of a test compound, and determining for each polypeptide-ligand pair whether the test compound inhibits binding between the immobilized polypeptide and the corresponding ligand.

Typically the plurality is at least 5, and often at least 25, or at least 40 different PDZ proteins. In a preferred embodiment, the plurality of different ligands and the plurality of different PDZ proteins are from the same tissue or a particular class or type of cell, (e.g., a neuron). In a most preferred embodiment, the plurality of different PDZs represents a substantial fraction (e.g., at least 80%) of all of the PDZs known to be, or suspected of being, expressed in the tissue or cell(s), e.g., all of the PDZs known to be present in neurons (for example, at least 80%, at least 90% or all of the PDZs disclosed herein as being expressed in neuronal cells).

In one embodiment, the inhibition profile is determined as follows: A plurality (e.g., all known) PDZ domains expressed in a cell (e.g., neurons) are expressed as GST-fusion

other ligands tested. Thus, the binding may be designated as "specific" if the affinity of the PDZ to the particular PL is at least 2-fold that of the binding to other PLs in the plurality (e.g., present in that cell type). The binding is deemed "very specific" if the affinity is at least 10-fold higher than to any other PL in the plurality or, alternatively, at least 10-fold higher than to at least 90%, more often 95% of the other PLs in a defined plurality. Similarly, the binding is deemed "exceedingly specific" if it is at least 100-fold higher. Typically the plurality is at least 5 different ligands, more often at least 10.

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Certain embodiments are arrays which include a plurality, usually at least 5, 10, 25, or 50 PDZ proteins present in a particular cell of interest. In this context, "array" refers to an ordered series of immobilized polypeptides in which the identity of each polypeptide is associated with its location. In some embodiments the plurality of polypeptides are arrayed in a "common" area such that they can be simultaneously exposed to a solution (e.g., containing a ligand or test agent). For example, the plurality of polypeptides can be on a slide, plate or similar surface, which may be plastic, glass, metal, silica, beads or other surface to which proteins can be immobilized. In a different embodiment, the different immobilized polypeptides are situated in separate areas, such as different wells of multi-well plate (e.g., a 24-well plate, a 96-well plate, a 384 well plate, and the like). It will be recognized that a similar advantage can be obtained by using multiple arrays in tandem.

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For any known or suspected modulator (e.g., inhibitor) of a PDL-PL interaction(s), it is useful to know which interactions are inhibited (or augmented). For example, an agent that inhibits all PDZ-PL interactions in a cell (e.g., a neuron) will have different uses than an agent that inhibits only one, or a small number, of specific PDZ-PL interactions. The profile of PDZ interactions inhibited by a particular agent is referred to as the "inhibition profile" for the agent, and is described in detail below. The profile of PDZ interactions enhanced by a particular agent is referred to as the "enhancement profile" for the agent. It will be readily apparent to one of skill guided by the description of the inhibition profile how to determine the enhancement profile for an agent. The present invention provides methods for determining the PDZ interaction (inhibition/enhancement) profile of an agent in a single assay.

In one aspect, the invention provides a method for determining the PDZ-PL inhibition profile of a compound by providing (i) a plurality of different immobilized polypeptides, each of said polypeptides comprising a PDZ domain and a non-PDZ domain and (ii) a plurality of corresponding ligands, wherein each ligand binds at least one PDZ domain in (i), then contacting each of said immobilized polypeptides in (i) with a corresponding ligand in (ii) in the presence and absence of a test compound, and determining for each polypeptide-ligand pair whether the test compound inhibits binding between the immobilized polypeptide and the corresponding ligand.

Typically the plurality is at least 5, and often at least 25, or at least 40 different PDZ proteins. In a preferred embodiment, the plurality of different ligands and the plurality of different PDZ proteins are from the same tissue or a particular class or type of cell, (e.g., a neuron). In a most preferred embodiment, the plurality of different PDZs represents a substantial fraction (e.g., at least 80%) of all of the PDZs known to be, or suspected of being, expressed in the tissue or cell(s), e.g., all of the PDZs known to be present in neurons (for example, at least 80%, at least 90% or all of the PDZs disclosed herein as being expressed in neuronal cells).

In one embodiment, the inhibition profile is determined as follows: A plurality (e.g., all known) PDZ domains expressed in a cell (e.g., neurons) are expressed as GST-fusion

proteins and immobilized without altering their ligand binding properties as described *supra*. For each PDZ domain, a labeled ligand that binds to this domain with a known affinity is identified. If the set of PDZ domains expressed in neurons is denoted by {P1...Pn}, any given PDZ domain Pi binds a (labeled) ligand Li with affinity K_di. To determine the inhibition profile for a test agent "compound X" the G assay (*supra*) can be performed as follows in 96-well plates with rows A-H and columns 1-12. Column 1 is coated with P1 and washed. The corresponding ligand L1 is added to each washed coated well of column 1 at a concentration 0.5 K_d1 with (rows B, D, F, H) or without (rows A, C, E, F) between about 1 and about 1000 μM) of test compound X. Column 2 is coated with P2, and L2 (at a concentration 0.5 K_d2) is added with or without inhibitor X. Additional PDZ domains and ligands are similarly tested.

Compound X is considered to inhibit the binding of Li to Pi if the average signal in the wells of column i containing X is less than half the signal in the equivalent wells of the column lacking X. Thus, in this single assay one determines the full set of neural PDZs that are inhibited by compound X.

In some embodiments, the test compound X is a mixture of compounds, such as the product of a combinatorial chemistry synthesis as described *supra*. In some embodiments, the test compound is known to have a desired biological effect, and the assay is used to determine the mechanism of action (i.e., if the biological effect is due to modulating a PDZ-PL interaction).

It will be apparent that an agent that modulates only one, or a few PDZ-PL interactions, in a panel (e.g., a panel of all known PDZs in neurons, a panel of at least 10, at least 20 or at least 50 PDZ domains) is a more specific modulator than an agent that modulate many or most interactions. Typically, an agent that modulates less than 20% of PDZ domains in a panel (e.g., **TABLE 5**) is deemed a "specific" inhibitor, less than 6% a "very specific" inhibitor, and a single PDZ domain a "maximally specific" inhibitor.

It will be recognized that high specificity modulators of PDZ-PL interactions represent potentially more valuable drug targets for achieving a desired biological effect. The ability of an inhibitor or enhancer to act with "maximal specificity" is most desirable.

In one embodiment, the assays of the invention can be used to determine a maximally specific modulator of the interaction between a TRP channel and a PDZ domain.

In a preferred embodiment, the assays of the invention are used to identify a maximally specific modulator of the interaction between TRPM7 and a PDZ domain.

It will also be appreciated that "compound X" may be a composition containing mixture of compounds (e.g., generated using combinatorial chemistry methods) rather than a single compound.

Several variations of this assay are contemplated:

In some alternative embodiments, the assay above is performed using varying concentrations of the test compound X, rather than fixed concentration. This allows determination of the Ki of the X for each PDZ as described above.

In an alternative embodiment, instead of pairing each PDZ Pi with a specific labeled ligand Li, a mixture of different labeled ligands is created that such that for every PDZ at least one of the ligands in the mixture binds to this PDZ sufficiently to detect the binding in the G assay. This mixture is then used for every PDZ domain.

In one embodiment, compound X is known to have a desired biological effect, but the chemical mechanism by which it has that effect is unknown. The assays of the invention can then be used to determine if compound X has its effect by binding to a PDZ domain.

In one embodiment, PDZ-domain containing proteins are classified in to groups based on their biological function, e.g. into those that regulate apoptosis versus those that regulate transcription. An optimal inhibitor of a particular function (e.g., including but not limited to an anti-apoptotic agent, an anti-T cell activation agent, cell-cycle control, vesicle transport, etc.) will inhibit multiple PDZ-ligand interactions involved in the function (e.g., apoptosis, activation) but few other interactions. Thus, the assay is used in one embodiment in screening and design of a drug that specifically blocks a particular function. For example, an agent designed to block apoptosis might be identified because, at a given concentration, the agent inhibits 2 or more PDZs involved in apoptosis but fewer than 3 other PDZs, or that inhibits PDZs involved in apoptosis with a K_i > 10-fold better than for other PDZs. Thus, the invention provides a method for identifying an agent that inhibits a first selected PDZ-PL interaction or plurality of interactions but does not inhibit a second selected PDZ-PL interaction or plurality of interactions. The two (or more) sets of interactions can be selected on the basis of the known biological function of the PDZ proteins, the tissue specificity of the PDZ proteins, or any other

criteria. Moreover, the assay can be used to determine effective doses (i.e., drug concentrations) that result in desired biological effects while avoiding undesirable effects.

3. Side Effects of PDZ-PL Modulator Interactions

In a related embodiment, the invention provides a method for determining likely side effects of a therapeutic that inhibits PDZ-ligand interactions. The method entails identifying those target tissues, organs or cell types that express PDZ proteins and ligands that are disrupted by a specified inhibitor. If, at a therapeutic dosage, a drug intended to have an effect in one organ system (e.g., central nervous system) disrupts PDZ-PL interactions in a different system (e.g., hematopoietic system) it can be predicted that the drug will have effects ("side effects") on the second system. It will be apparent that the information obtained from this assay will be useful in the rational design and selection of drugs that do not have the side-effect.

In one embodiment, for example, a comprehensive PDZ protein set is obtained. A "perfectly comprehensive" PDZ protein set is defined as the set of all PDZ proteins expressed in the subject animal (e.g., humans). A comprehensive set may be obtained by analysis of, for example, the human genome sequence. However, a "perfectly comprehensive" set is not required and any reasonably large set of PDZ domain proteins (e.g., the set of all known PDZ proteins; or the set listed in **TABLE 5**) will provide valuable information.

In one embodiment, the method involves some of all of the following steps:

a) For each PDZ protein, determine the tissues in which it is highly expressed. This can be done experimentally although the information generally will be available in the scientific literature;

b) For each PDZ protein (or as many as possible), identify the cognate PL(s) bound by the PDZ protein;

c) Determine the K_i at which the test agent inhibits each PDZ-PL interaction, using the methods described *supra*;

d) From this information it is possible to calculate the pattern of PDZ-PL interactions disrupted at various concentrations of the test agent.

By correlating the set of PDZ-PL interactions disrupted with the expression pattern of the members of that set, it will be possible to identify the tissues likely affected by the agent.

Additional steps can also be carried out, including determining whether a specified tissue or cell type is exposed to an agent following a particular route of administration. This can be determined using basis pharmacokinetic methods and principles.

4. Modulation of Activities

The PDZ binding moieties and inhibitors described herein that disrupt PDZ:PL protein interactions can be used to modulate biological activities or functions of cells (e.g., neurons). These agents can also be utilized to treat diseases and conditions in human and nonhuman animals (e.g., experimental models). Exemplary biological activities are listed *supra.*

When administered to patients, the compounds of the invention (e.g., PL-PDZ interaction inhibitors) are useful for treating (ameliorating symptoms of) a variety of neurological disorders, including those associated with some type of injury to neuronal cells or the death of neurons. Such disorders include, but are not limited to, stroke, ischemia, brain traumas and chronic pain. Certain inhibitors can also be used to treat other types of neuorological disorders like Alzheimer's disease, epilepsy, Parkinson's disease, Huntington's disease, motor neuron diseases and inherited ataxias.

Some other inhibitors can be utilized to treat other disease types, including, for instance, inflammatory and humoral immune responses, e.g., inflammation, allergy (e.g., systemic anaphylaxis, hypersensitivity responses, drug allergies, insect sting allergies); infectious diseases (e.g., viral infection, such as HIV, measles, parainfluenza, virus-mediated cell fusion,), and ischemia (e.g., post-myocardial infarction complications, joint injury, kidney, scleroderma).

VII. Antagonists of PDZ-PL Interactions

As described herein, interactions between PDZ proteins and PL proteins in cells (e.g., neurons) may be disrupted or inhibited by the administration of inhibitors or antagonists. Inhibitors can be identified using screening assays described herein. In embodiment, the motifs disclosed herein are used to design inhibitors. In some embodiments, the antagonists of the invention have a structure (e.g., peptide sequence) based on the C-terminal residues of PL-domain proteins listed in **TABLE 2** and **TABLE 4**. In some embodiments, the antagonists of the invention have a structure (e.g., peptide sequence) based on a PL motif disclosed herein.

The PDZ/PL antagonists and antagonists of the invention can be any of a large variety of compounds, both naturally occurring and synthetic, organic and inorganic, and including polymers (e.g., oligopeptides, polypeptides, oligonucleotides, and polynucleotides), small molecules, antibodies, sugars, fatty acids, nucleotides and nucleotide analogs, analogs of naturally occurring structures (e.g., peptide mimetics, nucleic acid analogs, and the like), and numerous other compounds. Although, for convenience, the present discussion primarily refers antagonists of PDZ-PL interactions, it will be recognized that PDZ-PL interaction agonists can also be use in the methods disclosed herein.

In one aspect, the peptides and peptide mimetics or analogues of the invention contain an amino acid sequence that binds a PDZ domain in a cell of interest. In one embodiment, the antagonists comprise a peptide that has a sequence corresponding to the carboxy-terminal sequence of a PL protein listed in **TABLE 2** or **TABLE 4**, e.g., a peptide listed **TABLE 2**. Typically, the peptide comprises at least the C-terminal two (3), three (3) or four (4) residues of the PL protein, and often the inhibitory peptide comprises more than four residues (e.g., at least five, six, seven, eight, nine, ten, twelve or fifteen residues) from the PL protein C-terminus.

In some embodiments, the inhibitor is a peptide, e.g., having a sequence of a PL C-terminal protein sequence.

In some embodiments, the antagonist is a fusion protein comprising such a sequence. Fusion proteins containing a transmembrane transporter amino acid sequence can be used to facilitate transport of the inhibitor into a cell.

In some embodiments, the inhibitor is conserved variant of the PL C-terminal protein sequence having inhibitory activity.

In some embodiments, the antagonist is a peptide mimetic of a PL C-terminal sequence.

In some embodiments, the inhibitor is a small molecule (i.e., having a molecular weight less than 1 kDa).

A. Polypeptide Antagonists

1. Inhibitors with a PL Sequence

One class of inhibitors or antagonists that are provided comprise a peptide that has a sequence of a PL protein carboxy-terminus listed in **TABLE 4**. The PL protein carboxy-terminus sequences can be considered as the "core PDZ motif sequence" because of the ability of the short sequence from the carboxy terminus to interact with the PDZ domain. For example, in some inhibitors the "core PDZ motif sequence" or simply the "PL sequence" contains the last 2, 3 or 4 C-terminus amino acids. In other instances, however, the core PDZ motif comprises more than 2-4 residues (e.g., at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 residues) from the PL protein C-terminus. For some inhibitors, the PDZ motif sequence peptide is from 4-15 amino acids in length. Other inhibitors have a PDZ motif sequence that is 6-10 amino acids in length, or 3-8 amino acids in length, or 3-7 amino acids in length. Certain inhibitors have a PDZ motif sequence that is 8 amino acids in length. Although the residues shared by the inhibitory peptide and the PL protein are often found at the C-terminus of the peptide, some inhibitors incorporate a PL sequence that is located in an internal region of a PL protein. Similarly, in some cases, the inhibitory peptide comprises residues from a PL sequence that is near, but not at the C-terminus of a PL protein (see, Gee et al., 1998, J Biol. Chem. 273:21980-87). Another set of inhibitors are based upon the identification of amino acid sequences that specifically disrupt binding between NMDAR proteins and PSD-95. This particular class of inhibitors are polypeptides that share the following characteristics: 1) a size ranging from 3-20 amino acids in length (although somewhat longer polypeptides can be used), and 2) a C-terminal consensus sequence of X-L/I/V-X-V/L/A (the slash separates different amino acids that can appear at a given position).

Specific examples of peptides which are useful as inhibitors of the interaction between TRPM7 and the PDZ domains RIM-2 d1, INADL d3, ZO-1 d2, PAR3 d3, and syntrophin1 alpha d1 are shown in TABLES 10 and 11 Variants of these peptides are which bind PDZ domains RIM-2 d1, INADL d3, ZO-1 d2, PAR3 d3, and syntrophin1 alpha d1 are also useful as inhibitors.

Table 10 - **titration of peptides with Z0-1 d2 and INADL d3**

Peptide #	Peptide Sequence	SEQ ID NO:	PDZ domain	EC50, uM	error of fit , EC50, uM	ODmax (450 nm)
1791	biotin-YGRKKRRQRRRAVAATSINL	261	ZO-1 d2	< 0.1	ND	2.50
1791	biotin-YGRKKRRQRRRAVAATSINL	261	INADL d3	ND	ND	0.09
1829	biotin-YGRKKRRQRRRARSDRTIWA	262	ZO-1 d2	0.007	0.0027	2.90
1829	biotin-YGRKKRRQRRRARSDRTIWA	262	INADL d3	ND	ND	1.40
1830	biotin-YGRKKRRQRRRARSDRTIIA	263	ZO-1 d2	< 0.02	ND	1.80
1830	biotin-YGRKKRRQRRRARSDRTIIA	263	INADL d3	< 2	ND	1.20
1832	biotin-YGRKKRRQRRRSRTDRKYWA	264	ZO-1 d2	< 0.02	ND	2.10
1832	biotin-YGRKKRRQRRRSRTDRKYWA	264	INADL d3	0.20	0.023	0.60
1836	biotin-YGRKKRRQRRRARGDRKIRV	265	ZO-1 d2	< 0.01	ND	2.60
1836	biotin-YGRKKRRQRRRARGDRKIRV	265	INADL d3	ND	ND	0.30
1837	biotin-YGRKKRRQRRRARTDRKVEV	266	ZO-1 d2	< 0.02	ND	1.80
1837	biotin-YGRKKRRQRRRARTDRKVEV	266	INADL d3	ND	ND	0.37
1838	biotin-YGRKKRRQRRRARGDRKYIV	267	ZO-1 d2	< 0.1	ND	2.60
1838	biotin-YGRKKRRQRRRARGDRKYIV	267	INADL d3	< 0.1	ND	2.60
1839	biotin-YGRKKRRQRRRSRTDRKYQI	268	ZO-1 d2	< 0.02	ND	2.50
1839	biotin-YGRKKRRQRRRSRTDRKYQI	268	INADL d3	0.13	0.007	1.60
1841	biotin-YGRKKRRQRRRARGDRKVPV	269	ZO-1 d2	< 0.02	ND	2.10
1841	biotin-YGRKKRRQRRRARGDRKVPV	269	INADL d3	ND	ND	0.20
1842	biotin-YGRKKRRQRRRQDERRLIVL	270	ZO-1 d2	0.035	0.008	2.30
1842	biotin-YGRKKRRQRRRQDERRLIVL	270	INADL d3	< 1	ND	1.90
1843	biotin-YGRKKRRQRRRARGDRLVSL	271	ZO-1 d2	0.017	0.003	2.20
1843	biotin-YGRKKRRQRRRARGDRLVSL	271	INADL d3	< 2 uM	ND	1.20
1844	biotin-YGRKKRRQRRRARGTRLVWV	272	ZO-1 d2	< 0.02	ND	2.60
1844	biotin-YGRKKRRQRRRARGTRLVWV	272	INADL d3	< 0.02	ND	2.40
1845	biotin-YGRKKRRQRRRARGDRYRIV	273	ZO-1 d2	< 0.1	ND	2.30
1845	biotin-YGRKKRRQRRRARGDRYRIV	273	INADL d3	< 0.1	ND	2.20
1846	biotin-YGRKKRRQRRRSRTDRLEYV	274	INADL d3	ND	ND	1.50
1848	biotin-YGRKKRRQRRRARGDRTIIY	275	ZO-1 d2	< 0.02	ND	2.20
1848	biotin-YGRKKRRQRRRARGDRTIIY	275	INADL d3	~< 2	ND	1.20
1852	biotin-YGRKKRRQRRRKNKDKEYYV	276	ZO-1 d2	< 0.02	ND	2.60
1852	biotin-YGRKKRRQRRRKNKDKEYYV	276	INADL d3	< 0.02	ND	2.40
1854	biotin-YGRKKRRQRRRARGRRETWV	277	ZO-1 d2	ND	ND	2.80
1854	biotin-YGRKKRRQRRRARGRRETWV	277	INADL d3	< 0.02	ND	2.10
AA353	biotin-YGRKKRRQRRREKHFRETEV	278	TIP1	< 0.002	ND	3.00

TABLE 11 - titrations or binding assays of peptides with RIM-2 d1, INADL d3, ZO-1 d2, PAR3 d3, and syntrophin1 alpha d1

PDZ domain bound	Peptide Sequence	SEQ ID NO:
INADL D3	ATDYLVQPFMDQLAFHQFYI	280
INADL D3	DFRPSFKHILFRRARRGFRQ	282
INADL D3	ELLQFCRTPNPALKNGQYWV	285
INADL D3	ENLELPVNPSSVVSERISSV	286
INADL D3	FHSKTAGANTTDKELEVLSL	287
INADL D3	GRWTGRAMSAWKPTRRETEV	289
INADL D3	HAMNAAPRAMENAPALRTSH	290
INADL D3	HDFRRAFKKILARGDRKRIV	291
INADL D3	HHLVAQRDIRQFQLQHWLAI	292
INADL D3	HSCCNRARQERLQRRRETQV	293
INADL D3	ILNSIQVMRAQMNQIQSVEV	294
INADL D3	KHSRKSSSYSSSSTTVKTSY	296
INADL D3	KKKKQPGNSTKESESTNSVRLML	297
INADL D3	LASKSAEEGKQIPDSLSTDL	302
INADL D3	LAVLAYSITLVMLWSIWQYA	303
INADL D3	LNSCSNRRVYKKMPSIESDV	305
INADL D3	LQFHRGSRAQSFLQTETSVI	306
INADL D3	PGQPPKVKSEFNSYSLTGYV	308
INADL D3	PIPAGGCTFSGIFPTLTSPL	309
INADL D3	QDFRRAFRRILARPWTQTAW	312
INADL D3	RELVDRGEVRQFTLRHWLKV	315
INADL D3	RSGATIPLVGQDIIDLQTEV	318
INADL D3	SLIGPVQKEYQRELGKLSSP	319
INADL D3	SSKSKSSEESQTFFGLYKL	320
INADL D3	STDNLVRPFMDTLASHQLYI	323
INADL D3	TQGFPGPATWRRISSLESEV	327
INADL D3	TTNNNPNSAVNIKKIFTDV	328
INADL D3	VDPNSPAAKKKYVSYNNLVI	329
INADL D3	VHKVRNKFKAKCSLCRLYII	331
INADL D3	VPSDNIDSQGRNASTNDSLL	333
INADL D3	YGRKKRRQRRRARGDRKYIV	339
INADL D3	YGRKKRRQRRRARSDRTIIA	350
INADL D3	YGRKKRRQRRRARSDRTIWA	351
INADL D3	YGRKKRRQRRRAVAATSANL	355
INADL D3	YGRKKRRQRRREYLGLDVPV	360
INADL D3	YGRKKRRQRRRSRTDRKYQI	373
INADL D3	YGRKKRRQRRRSRTDRKYWA	374
INADL D3	YSATYSELEDPGEMSPPIDL	378
PAR3 D3	ATDYLVQPFMDQLAFHQFYI	280
PAR3 D3	DFRPSFKHILFRRARRGFRQ	282
PAR3 D3	DGGARTEDEVQSYPSKHDYV	283
PAR3 D3	DTLLLTENEGDKTEEQVSYV	284
PAR3 D3	ELLQFCRTPNPALKNGQYWV	285
PAR3 D3	FHSKTAGANTTDKELEVLSL	287
PAR3 D3	HDFRRAFKKILARGDRKRIV	291

PAR3 D3	HHLVAQRDIRQFQLQHWLAI	292
PAR3 D3	HSCCNRARQERLQRRRETQV	293
PAR3 D3	ILNSIQVMRAQMNQIQSVEV	294
PAR3 D3	KAGYRAPRSYPKSNSSKEYV	295
PAR3 D3	KHSRKSSSYSSSSTTVKTSY	296
PAR3 D3	KKKKQPGNSTKESESTNSVRLML	297
PAR3 D3	KTMPAAMFRLLTGQETPLYI	298
PAR3 D3	KTMPAAMYRLLTAQEQPVYI	299
PAR3 D3	KTMPAATYRLLTGQEQPVYL	300
PAR3 D3	KYSAPRRPTATGDYDKKNYV	301
PAR3 D3	LASKSAEEGKQIPDSLSTDL	302
PAR3 D3	LAVLAYSITLVMLWSIWQYA	303
PAR3 D3	LERTSSVSPSTAEPELSIVF	304
PAR3 D3	LQFHRGSRAQSFLQTETSVI	306
PAR3 D3	PGQPPKVKSEFNSYSLTGYV	308
PAR3 D3	PIPAGGCTFSGIFPTLTSPL	309
PAR3 D3	QDFRRAFRRILARPWTQTAW	312
PAR3 D3	QGDPALQDAGDSSRKEYFI	313
PAR3 D3	RELVDRGEVRQFTLRHWLKV	315
PAR3 D3	SSKSKSSEESQTFFGLYKL	320
PAR3 D3	SSPDSSYQGKGFVMSRAMYV	321
PAR3 D3	STDNLVRPFMDTLASHQLYI	323
PAR3 D3	VDPNSPAAKKKYVSYNNLVI	329
PAR3 D3	VHKVRNKFKAKCSLCRLYII	331
PAR3 D3	YGRKKRRQRRRAVAATSANL	355
PAR3 D3	YGRKKRRQRRRAVAATSINL	356
PAR3 D3	YGRKKRRQRRREYLGLDVPV	360
PAR3 D3	YGRKKRRQRRRGASADSTQA	361
PAR3 D3	YGRKKRRQRRRKLSSIESDV	363
PAR3 D3	YGRKKRRQRRRNDNIALLVQ	366
PAR3 D3	YGRKKRRQRRRSEGVPDLLV	372
RIM2 D1	DFRPSFKHILFRRARRGFRQ	282
RIM2 D1	FHSKTAGANTTDKELEVLSL	287
RIM2 D1	HDFRRAFKKILARGDRKRIV	291
RIM2 D1	HHLVAQRDIRQFQLQHWLAI	292
RIM2 D1	KHSRKSSSYSSSSTTVKTSY	296
RIM2 D1	KKKKQPGNSTKESESTNSVRLML	297
RIM2 D1	LAVLAYSITLVMLWSIWQYA	303
RIM2 D1	NYKLNTDHAGSNDNIALLVQ	307
RIM2 D1	QDFRRAFRRILARPWTQTAW	312
RIM2 D1	RELVDRGEVRQFTLRHWLKV	315
RIM2 D1	SSKSKSSEESQTFFGLYKL	320
RIM2 D1	SSSRRDSSWSETSEASYSGL	322
RIM2 D1	VDPNSPAAKKKYVSYNNLVI	329
RIM2 D1	VHKVRNKFKAKCSLCRLYII	331
RIM2 D1	YGRKKRRQRRRARGDRKIRV	334
RIM2 D1	YGRKKRRQRRRARGDRKKIV	335
RIM2 D1	YGRKKRRQRRRARGDRKRIV	335
RIM2 D1	YGRKKRRQRRRARGDRKRWA	336

RIM2 D1	YGRKKRRQRRRARGDRKRWL	337
RIM2 D1	YGRKKRRQRRRARGDRKVPV	338
RIM2 D1	YGRKKRRQRRRARGDRKYIV	339
RIM2 D1	YGRKKRRQRRRARGDRLEIV	340
RIM2 D1	YGRKKRRQRRRARGDRLVSL	341
RIM2 D1	YGRKKRRQRRRARGDRRRIV	342
RIM2 D1	YGRKKRRQRRRARGDRTIIY	343
RIM2 D1	YGRKKRRQRRRARGDRYRIV	344
RIM2 D1	YGRKKRRQRRRARGDVRLML	345
RIM2 D1	YGRKKRRQRRRARGRRETWV	346
RIM2 D1	YGRKKRRQRRRARGTRLVWV	346
RIM2 D1	YGRKKRRQRRRARSDRGIWA	347
RIM2 D1	YGRKKRRQRRRARSDRKRIA	348
RIM2 D1	YGRKKRRQRRRARSDRKRIV	349
RIM2 D1	YGRKKRRQRRRARSDRTIIA	350
RIM2 D1	YGRKKRRQRRRARSDRTIWA	351
RIM2 D1	YGRKKRRQRRRARTDRKVEV	352
RIM2 D1	YGRKKRRQRRRAVAAASANL	353
RIM2 D1	YGRKKRRQRRRAVAATGIWA	354
RIM2 D1	YGRKKRRQRRRAVAATSANL	355
RIM2 D1	YGRKKRRQRRRAVAATSINL	356
RIM2 D1	YGRKKRRQRRRAVAATYSNL	357
RIM2 D1	YGRKKRRQRRREKHFRETEV	359
RIM2 D1	YGRKKRRQRRREYLGLDVPV	360
RIM2 D1	YGRKKRRQRRRGASADSTQA	361
RIM2 D1	YGRKKRRQRRRGMTSSSSVV	362
RIM2 D1	YGRKKRRQRRRKNKDKEYYV	364
RIM2 D1	YGRKKRRQRRRLQRRRETQV	365
RIM2 D1	YGRKKRRQRRRNDNIALLVQ	366
RIM2 D1	YGRKKRRQRRRQDEEEGIWA	367
RIM2 D1	YGRKKRRQRRRQDEEEGIWS	368
RIM2 D1	YGRKKRRQRRRQDEEETIWA	369
RIM2 D1	YGRKKRRQRRRQDERRLIVL	370
RIM2 D1	YGRKKRRQRRRQDERVETRV	371
RIM2 D1	YGRKKRRQRRRSRTDRKYQI	373
RIM2 D1	YGRKKRRQRRRSRTDRKYWA	374
RIM2 D1	YGRKKRRQRRRSRTDRLEYV	375
RIM2 D1	YGRKKRRQRRRSRTVREIWA	376
RIM2 D1	YGRKKRRQRRRSVTSTSINL	377
Syntrophin 1 alpha D1	AAGGRSARGGRLQGRRETAL	279
Syntrophin 1 alpha D1	ATDYLVQPFMDQLAFHQFYI	280
Syntrophin 1 alpha D1	AVGGRPARGGRLQGRRQTQV	281
Syntrophin 1 alpha D1	DFRPSFKHILFRRARRGFRQ	282
Syntrophin 1 alpha D1	DTLLLTENEGDKTEEQVSYV	284
Syntrophin 1 alpha D1	FNGSSNGHVYEKLSSIESDV	288
Syntrophin 1 alpha D1	GRWTGRAMSAWKPTRRETEV	289
Syntrophin 1 alpha D1	HDFRRAFKKILARGDRKRIV	291
Syntrophin 1 alpha D1	HHLVAQRDIRQFQLQHWLAI	292
Syntrophin 1 alpha D1	HSCCNRARQERLQRRRETQV	293

Syntrophin 1 alpha D1	ILNSIQVMRAQMNQIQSVEV	294
Syntrophin 1 alpha D1	KKKKQPGNSTKESESTNSVRLML	297
Syntrophin 1 alpha D1	LAVLAYSITLVMLWSIWQYA	303
Syntrophin 1 alpha D1	PIPAGGCTFSGIFPTLTSPL	309
Syntrophin 1 alpha D1	PYSELNYETSHYPASPDSWV	311
Syntrophin 1 alpha D1	QDFRRAFRRILARPWTQTAW	312
Syntrophin 1 alpha D1	QISPGGLEPPSEKHFRETEV	314
Syntrophin 1 alpha D1	RELVDRGEVRQFTLRHWLKV	315
Syntrophin 1 alpha D1	SSKSKSSEESQTFFGLYKL	320
Syntrophin 1 alpha D1	TFKGTPTAENPEYLGLDVPV	325
Syntrophin 1 alpha D1	TGSALQAWRHTSRQATESTV	326
Syntrophin 1 alpha D1	TQGFPGPATWRRISSLESEV	327
Syntrophin 1 alpha D1	VHDAESSDEDGYDWGPATDL	330
Syntrophin 1 alpha D1	VHKVRNKFKAKCSLCRLYII	331
Syntrophin 1 alpha D1	VPGALDYAAFSSALYGESDL	332
Syntrophin 1 alpha D1	YGRKKRRQRRRKLSSIESDV	363
Syntrophin 1 alpha D1	YGRKKRRQRRRNDNIALLVQ	366
ZO-1 D2	DFRPSFKHILFRRARRGFRQ	282
ZO-1 D2	ELLQFCRTPNPALKNGQYWV	285
ZO-1 D2	FHSKTAGANTTDKELEVLSL	287
ZO-1 D2	HDFRRAFKKILARGDRKRIV	291
ZO-1 D2	HHLVAQRDIRQFQLQHWLAI	292
ZO-1 D2	HSCCNRARQERLQRRRETQV	293
ZO-1 D2	ILNSIQVMRAQMNQIQSVEV	294
ZO-1 D2	KHSRKSSSYSSSSTTVKTSY	296
ZO-1 D2	KKKKQPGNSTKESESTNSVRLML	297
ZO-1 D2	LAVLAYSITLVMLWSIWQYA	303
ZO-1 D2	LNSCSNRRVYKKMPSIESDV	305
ZO-1 D2	NYKLNTDHAGSNDNIALLVQ	307
ZO-1 D2	PSSRASSRASSRPRPDDLEI	310
ZO-1 D2	QDFRRAFRRILARPWTQTAW	312
ZO-1 D2	RELVDRGEVRQFTLRHWLKV	315
ZO-1 D2	RRRRRRRGNTTDKELEVLSL	316
ZO-1 D2	RRRRRRRGTNPAVAATSANL	317
ZO-1 D2	SSKSKSSEESQTFFGLYKL	320
ZO-1 D2	SSSRRDSSWSETSEASYSGL	322
ZO-1 D2	TEGNESSEATSPVNAIYSLA	324
ZO-1 D2	TTNNNPNSAVNIKKIFTDV	328
ZO-1 D2	VDPNSPAAKKKYVSYNNLVI	329
ZO-1 D2	VHKVRNKFKAKCSLCRLYII	331
ZO-1 D2	YGRKKRRQRRRARGDRKRIV	335
ZO-1 D2	YGRKKRRQRRRARGDRKRWA	336
ZO-1 D2	YGRKKRRQRRRARGDRKRWL	337
ZO-1 D2	YGRKKRRQRRRARGDRKVPV	338
ZO-1 D2	YGRKKRRQRRRARGDRLVSL	341
ZO-1 D2	YGRKKRRQRRRARGDRTIIY	343
ZO-1 D2	YGRKKRRQRRRARGDRYRIV	344
ZO-1 D2	YGRKKRRQRRRARGRRETWV	346
ZO-1 D2	YGRKKRRQRRRARGTRLVWV	346

ZO-1 D2	YGRKKRRQRRRARSDRGIWA	347
ZO-1 D2	YGRKKRRQRRRARSDRTIIA	350
ZO-1 D2	YGRKKRRQRRRARSDRTIWA	351
ZO-1 D2	YGRKKRRQRRRAVAAASANL	353
ZO-1 D2	YGRKKRRQRRRAVAATSANL	355
ZO-1 D2	YGRKKRRQRRRAVAATSINL	356
ZO-1 D2	YGRKKRRQRRRAVAATYSNL	357
ZO-1 D2	YGRKKRRQRRRDKELEVLSL	358
ZO-1 D2	YGRKKRRQRRREKHFRETEV	359
ZO-1 D2	YGRKKRRQRRREYLGLDVPV	360
ZO-1 D2	YGRKKRRQRRRGASADSTQA	361
ZO-1 D2	YGRKKRRQRRRKLSSIESDV	363
ZO-1 D2	YGRKKRRQRRRKNKDKEYYV	364
ZO-1 D2	YGRKKRRQRRRNDNIALLVQ	366
ZO-1 D2	YGRKKRRQRRRQDEEEGIWA	367
ZO-1 D2	YGRKKRRQRRRQDERRLIVL	370
ZO-1 D2	YGRKKRRQRRRSEGVPDLLV	372
ZO-1 D2	YGRKKRRQRRRSRTDRKYWA	374
ZO-1 D2	YVYSRVKNLNSSEGVPDLLV	379

These specific examples should not be considered as limiting but simply illustrative of inhibitors having the general characteristics listed above.

As described in greater detail below, short PL peptides, such as just described can be used in the rational design of other small molecules with similar properties according to established techniques.

Core PDZ motif sequences/PL sequences such as those just listed can optionally be joined to additional amino acids at their amino terminus to further increase binding affinity and/or stability and/or transportability into cells. These additional sequences located at the amino terminus can be from the natural sequence of a neuronal cell surface receptor or from other sources. The PDZ motif sequence and additional N-terminal sequences can optionally be joined by a linker. The additional amino acids can also be conservatively substituted. The total peptide length (i.e., core PDZ motif sequence plus optional N-terminal segment) can be of a variety of lengths (e.g., at least 2, 3, 4, 5, 6, 8, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100 or more amino acids). Typically, the overall length is in the range of 30-40 amino acids) For those inhibitors in which additional sequences are attached at the N-terminus of the core PDZ motif sequence (PL sequence), the overall structure is thus: N-terminal segment - core PDZ motif sequence (PL sequence), or N-terminal segment - linker - core PDZ motif sequence (PL

sequence). As discussed further below, one useful class of proteins that can be fused to the core PDZ motifs or PL sequences are transmembrane transporter peptides. These peptides can be fused to the inhibitory sequences to facilitate transport into a target cell (e.g., neuron). Further details are provided below. Purification tags that are known in the art can also optionally be fused to the N-terminus of the PL sequence.

2. Inhibitors with a PDZ-Domain Polypeptide

Some of the inhibitors that are provided contain all or a portion of a PDZ binding domain rather than containing a PL sequence. The PDZ-domain sequence included in these inhibitors is selected to mimic (i.e., have similar binding characteristics) of the PDZ domain in the PDZ protein of interest (i.e., the PDZ protein whose binding interaction with a PL protein one seeks to disrupt). The PDZ-domain sequence is long enough to include at least enough of the PDZ domain such that the resulting polypeptide inhibitor can effectively bind to the cognate PL protein. This typically means that the PDZ-domain sequence is at least 50, 55, 60, 65, 70, 75, 80, 85, 90 or more amino acids long. But certain inhibitors can include the entire PDZ-domain, or even additional amino acids from the PDZ protein that extend beyond the PDZ-domain.

3. Optional Features of Inhibitors

Polypeptide inhibitors such as those just described can optionally be derivatized (e.g., acetylated, phosphorylated and/or glycoslylated) to improve the binding affinity of the inhibitor, to improve the ability of the inhibitor to be transported across a cell membrane or to improve stability. As a specific example, for inhibitors in which the third residue from the C-terminus is S, T or Y, this residue can be phosphorylated prior to the use of the peptide.

The polypeptide inhibitors can also optionally be linked directly or via a linker to a transmembrane transporter peptide. Specific examples of these sequences are described in the section on formulation and administration of the polypeptides of the invention. But certain polypeptide inhibitors do not include a transporter peptide.

B. Peptide Variants

Having identified PDZ binding peptides and PDZ-PL interaction inhibitory sequences, variations of these sequences can be made and the resulting peptide variants can be

tested for PDZ domain binding or PDZ-PL inhibitory activity. In embodiments, the variants have the same or a different ability to bind a PDZ domain as the parent peptide. Typically, such amino acid substitutions are conservative, i.e., the amino acid residues are replaced with other amino acid residues having physical and/or chemical properties similar to the residues they are replacing. Preferably, conservative amino acid substitutions are those wherein an amino acid is replaced with another amino acid encompassed within the same designated class.

C. Peptide Mimetics

Having identified PDZ binding peptides and PDZ-PL interaction inhibitory sequences, peptide mimetics can be prepared using routine methods, and the inhibitory activity of the mimetics can be confirmed using the assays of the invention. Thus, in some embodiments, the antagonist is a peptide mimetic of a PL C-terminal sequence. The skilled artisan will recognize that individual synthetic residues and polypeptides incorporating mimetics can be synthesized using a variety of procedures and methodologies, which are well described in the scientific and patent literature, e.g., *Organic Syntheses Collective Volumes*, Gilman et al. (Eds) John Wiley & Sons, Inc., NY. Polypeptides incorporating mimetics can also be made using solid phase synthetic procedures, as described, e.g., by Di Marchi, et al., U.S. Patent No. 5,422,426. Mimetics of the invention can also be synthesized using combinatorial methodologies. Various techniques for generation of peptide and peptidomimetic libraries are well known, and include, e.g., multipin, tea bag, and split-couple-mix techniques; see, e.g., al-Obeidi (1998) Mol. Biotechnol. 9:205-223; Hruby (1997) Curr. Opin. Chem. Biol. 1:114-119; Ostergaard (1997) Mol. Divers. 3:17-27; Ostresh (1996) Methods Enzymol. 267:220-234.

D. Small Molecules

In some embodiments, the inhibitor is a small molecule (i.e., having a molecular weight less than 1 kDa). Methods for screening small molecules are well known in the art and include those described *supra*.

E. Binding Affinity

Regardless of type, the inhibitors generally have an EC_{50} of less than 50 μm. Some inhibitors have an EC_{50} of less than 10 μM, others have an EC_{50} of 1 μM, and still others an EC_{50} of less than 100 nM. The inhibitors typically have an EC_{50} value of 20-100 nM.

VIII. Uses of PDZ Domain Binding and Antagonist Compounds

Because the inhibitors that are described herein are useful in interfering with binding between certain PDZ and PL proteins in neurons (e.g., the TRPM7/PDZ interaction), the inhibitors can be utilized in the treatment of a variety of biological processes in neuron cells. For instance, the inhibitors can be utilized to treat problems associated with excitotoxicity and apoptosis occasioned by neuronal damage. The inhibitors can also be utilized to treat various neurological diseases, including those associated with stroke and ischemia. Specific examples of neurological diseases that can be treated with certain inhibitors include, Alzheimer's disease, epilepsy, Parkinson's disease, Huntington's disease, motor neuron diseases and inherited ataxias.

Because PDZ proteins are involved in a number of biological functions besides involvement in excitotoxicity responses, some of the inhibitors that are provided can be used in the treatment of other conditions and activities correlated with the PDZ:PL protein interactions described herein. Examples of such activities include, but are not limited to, organization and regulation of multiprotein complexes, vesicular trafficking, tumor suppression, protein sorting, establishment of membrane polarity, apoptosis, regulation of immune response and organization of synapse formation. In general, PDZ proteins have a common function of facilitating the assembly of multi-protein complexes, often serving as a bridge between several proteins, or regulating the function of other proteins. Additionally, as also noted supra, these proteins are found in essentially all cell types.

Consequently, modulation of these interactions can be utilized to control a wide variety of biological conditions and physiological conditions. In particular, modulation of interactions such as those disclosed herein can be utilized to control movement of vesicles within a cell, inhibition of tumor formation, as well as in the treatment of immune disorders, neurological disorders, muscular disorders, and intestinal disorders.

Certain compounds which modulate binding of the PDZ proteins and PL proteins can be used to inhibit leukocyte activation, which is manifested in measurable events including but not limited to, cytokine production, cell adhesion, expansion of cell numbers, apoptosis and

cytotoxicity. Thus, some compounds of the invention can be used to treat diverse conditions associated with undesirable leukocyte activation, including but not limited to, acute and chronic inflammation, graft-versus-host disease, transplantation rejection, hypersensitivities and autoimmunity such as multiple sclerosis, rheumatoid arthritis, peridontal disease, systemic lupus erythematosis, juvenile diabetes mellitus, non-insulin-dependent diabetes, and allergies, and other conditions listed herein.

Thus, the invention also relates to methods of using such compositions in modulating leukocyte activation as measured by, for example, cytotoxicity, cytokine production, cell proliferation, and apoptosis.

IX. Formulation and Route of Administration

A. Introduction of Antagonists (e.g., Peptides and Fusion Proteins) into Cells

The inhibitors disclosed herein or identified using the screening methods that are provided can be used in the manufacture of a medicament or pharmaceutical composition. These can then be administered according to a number of different methods.

In one aspect, the PDZ-PL antagonists of the invention are introduced into a cell to modulate (i.e., increase or decrease) a biological function or activity of the cell. Many small organic molecules readily cross the cell membranes (or can be modified by one of skill using routine methods to increase the ability of compounds to enter cells, e.g., by reducing or eliminating charge, increasing lipophilicity, conjugating the molecule to a moiety targeting a cell surface receptor such that after interacting with the receptor). Methods for introducing larger molecules, e.g., peptides and fusion proteins are also well known, including, e.g., injection, liposome-mediated fusion, application of a hydrogel, conjugation to a targeting moiety conjugate endocytozed by the cell, electroporation, and the like).

In one embodiment, the antagonist or agent is a fusion polypeptide or derivatized polypeptide. A fusion or derivatized protein may include a targeting moiety that increases the ability of the polypeptide to traverse a cell membrane or causes the polypeptide to be delivered to a specified cell type (e.g., a neuron) preferentially or cell compartment (e.g., nuclear compartment) preferentially. Examples of targeting moieties include lipid tails, amino acid

sequences such as antennapoedia peptide or a nuclear localization signal (NLS; e.g., Xenopus nucleoplasmin Robbins et al., 1991, Cell 64:615).

In one embodiment of the invention, a peptide sequence or peptide analog determined to inhibit a PDZ domain-PL protein binding interaction as described herein is introduced into a cell by linking the sequence to an amino acid sequence that facilitates its transport through the plasma membrane (a "transmembrane transporter sequence"). The peptides of the invention may be used directly or fused to a transmembrane transporter sequence to facilitate their entry into cells. In the case of such a fusion peptide, each peptide may be fused with a heterologous peptide at its amino terminus directly or by using a flexible polylinker such as the pentamer G-G-G-G-S (SEQ ID NO:254) repeated 1 to 3 times. Such linker has been used in constructing single chain antibodies (scFv) by being inserted between V_H and V_L (Bird et al., 1988, *Science* 242:423-426; Huston et al., 1988, *Proc. Natl. Acad. Sci. U.S.A.* 85:5979-5883). The linker is designed to enable the correct interaction between two beta-sheets forming the variable region of the single chain antibody. Other linkers which may be used include Glu-Gly-Lys-Ser-Ser-Gly-Ser-Gly-Ser-Glu-Ser-Lys-Val-Asp (SEQ ID NO:255) (Chaudhary et al., 1990, *Proc. Natl. Acad. Sci. U.S.A.* 87:1066-1070) and Lys-Glu-Ser-Gly-Ser-Val-Ser-Ser-Glu-Gln-Leu-Ala-Gln-Phe-Arg-Ser-Leu-Asp (SEQ ID NO:256) (Bird et al., 1988, *Science* 242:423-426).

A number of peptide sequences have been described in the art as capable of facilitating the entry of a peptide linked to these sequences into a cell through the plasma membrane (Derossi et al., 1998, Trends in Cell Biol. 8:84). For the purpose of this invention, such peptides are collectively referred to as transmembrane transporter peptides. Examples of these peptides include, but are not limited to, tat derived from HIV (Vives et al., 1997, *J. Biol. Chem.* 272:16010; Nagahara et al., 1998, *Nat. Med.* 4:1449), antennapedia from Drosophila (Derossi et al., 1994, *J. Biol. Chem.* 261:10444), VP22 from herpes simplex virus (Elliot and D'Hare, 1997, Cell 88:223-233), complementarity-determining regions (CDR) 2 and 3 of anti-DNA antibodies (Avrameas et al., 1998, *Proc. Natl Acad. Sci. U.S.A.*, 95:5601-5606), 70 kDa heat shock protein (Fujihara, 1999, *EMBO J.* 18:411-419), transportan (Pooga et al., 1998, *FASEB J.* 12:67-77), penetratin, SynB1, SynB3, amphipathic model peptide, signal sequence-based peptides, and Arg, as described in Temsamani et al. (2004) Drug Discovery Today 9:1012-1019. In a preferred embodiment of the invention, a truncated HIV tat peptide having the sequence of YGRKKRRQRRR (SEQ ID NO:257) is used.

In some instances, a transmembrane transporter sequence is fused to a neuronal cell surface receptor carboxyl terminal sequence at its amino-terminus with or without a linker. Generally, the C-terminus of a PDZ motif sequence (PL sequence) is free to interact with a PDZ domain. The transmembrane transporter sequence can be used in whole or in part as long as it is capable of facilitating entry of the peptide into a cell.

In an alternate embodiment of the invention, a neuronal cell surface receptor C-terminal sequence can be used alone when it is delivered in a manner that allows its entry into cells in the absence of a transmembrane transporter sequence. For example, the peptide may be delivered in a liposome formulation or using a gene therapy approach by delivering a coding sequence for the PDZ motif alone or as a fusion molecule into a target cell.

The compounds of the invention can also be administered via liposomes, which serve to target the conjugates to a particular tissue, such as neural tissue, or targeted selectively to infected cells, as well as increase the half-life of the peptide composition. Liposomes include emulsions, foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions, lamellar layers and the like. In these preparations, the peptide to be delivered is incorporated as part of a liposome, alone or in conjunction with a molecule which binds to, e.g., a receptor prevalent among neural cells, such as monoclonal antibodies which bind to the NMDA Receptor. Thus, liposomes filled with a desired peptide or conjugate of the invention can be directed to the site of neural cells, where the liposomes then deliver the selected inhibitor compositions. Liposomes for use in the invention are formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of, e.g., liposome size, acid lability and stability of the liposomes in the blood stream. A variety of methods are available for preparing liposomes, as described in, e.g., Szoka et al., Ann. Rev. Biophys. Bioeng. 9:467 (1980), U.S. Patent Nos. 4,235,871, 4,501,728 and 4,837,028.

The targeting of liposomes using a variety of targeting agents is well known in the art (see, e.g., U.S. Patent Nos. 4,957,773 and 4,603,044). For targeting to the neural cells, a ligand to be incorporated into the liposome can include, e.g., antibodies or fragments thereof specific for cell surface determinants of the desired nervous system cells. A liposome suspension containing a peptide or conjugate may be administered intravenously, locally,

topically, etc. in a dose which varies according to, inter alia, the manner of administration, the conjugate being delivered, and the stage of the disease being treated.

In order to specifically deliver a PDZ motif sequence or PL sequence peptide into a specific cell type, the peptide can be linked to a cell-specific targeting moiety, which include but are not limited to, ligands for diverse neuron surface molecules such as growth factors, hormones and cytokines, neuronal receptors, ion transporters, as well as antibodies or antigen-binding fragments thereof. Since a large number of cell surface receptors have been identified in neurons, ligands or antibodies specific for these receptors may be used as cell-specific targeting moieties.

Antibodies are the most versatile cell-specific targeting moieties because they can be generated against any cell surface antigen. Monoclonal antibodies have been generated against neuron-specific markers. Antibody variable region genes can be readily isolated from hybridoma cells by methods well known in the art. However, since antibodies are assembled between two heavy chains and two light chains, it is preferred that a scFv be used as a cell-specific targeting moiety in the present invention. Such scFv are comprised of V_H and V_L domains linked into a single polypeptide chain by a flexible linker peptide.

The PDZ motif sequence (PL sequence) may be linked to a transmembrane transporter sequence and a cell-specific targeting moiety to produce a tri-fusion molecule. This molecule can bind to a neuron surface molecule, pass through the membrane and target PDZ domains. Alternatively, a PDZ motif sequence (PL sequence) may be linked to a cell-specific targeting moiety that binds to a surface molecule that internalizes the fusion peptide.

In another approach, microspheres of artificial polymers of mixed amino acids (proteinoids) have been used to deliver pharmaceuticals. For example, U.S. Patent No. 4,925,673 describes drug-containing proteinoid microsphere carriers as well as methods for their preparation and use. These proteinoid microspheres are useful for the delivery of a number of active agents. Also see, U.S. Patent Nos. 5,907,030 and 6,033,884, which are incorporated herein by reference.

B. Introduction of Polynucleotides into Cells

By introducing gene sequences into cells, gene therapy can be used to treat diseased cells (e.g., neuron cells that are associated with apoptosis or an excitotoxic response due

to a neuronal insult). In one embodiment, a polynucleotide that encodes a PL sequence peptide of the invention is introduced into a cell where it is expressed. The expressed peptide then inhibits the interaction of PDZ proteins and PL proteins in the cell. In another embodiment, the expression of a given protein would be suppressed, thus inhibiting its interactions with other proteins. In a specific embodiment, a polynucleotide that encoders an interfering RNA duplex is introduced into the cell. This results in RNA interference (RNAi), a process of post transcriptional gene silencing that inhibits, with high specificity, the expression of native genes in mammalian cells (Elbashir et al., 2001; Cullen, 2002). In a specific embodiment, the polynucleotides of the invention would suppress the expression of a given TRP channel such as TRPM7, or the expression of a protein with which the TRP channel interacts.

Examples of specific siRNAs against TRPM7 corresponded to coding regions 5152-5172, 5023-5043 and 1318-1338 ($siRNA_{TRPM7-1}$ to $siRNA_{TRPM7-3}$, respectively) relative to the first nucleotide of the start codon of murine TRPM7 (GenBank accession # AY032951). These siRNAs can reduce the expression levels of TRPM7 in cells (Figure 8) and have functional consequences (Figure 3). Thus, these sequences or related sequences could be used directly or with an appropriate delivery system to treat cells damaged by OGD or other cytodestructive disorders.

In one embodiment, the polypeptides of the invention are expressed in a cell by introducing a nucleic acid (e.g., a DNA expression vector or mRNA) encoding the desired protein or peptide into the cell. Expression can be either constitutive or inducible depending on the vector and choice of promoter. Methods for introduction and expression of nucleic acids into a cell are well known in the art and described herein.

In a specific embodiment, nucleic acids comprising a sequence encoding a peptide disclosed herein, are administered to a human subject. In this embodiment of the invention, the nucleic acid produces its encoded product that mediates a therapeutic effect. Any of the methods for gene therapy available in the art can be used according to the present invention. Exemplary methods are described below.

For general reviews of the methods of gene therapy, see Goldspiel et al., 1993, Clinical Pharmacy 12:488-505; Wu and Wu, 1991, Biotherapy 3:87-95; Tolstoshev, 1993, Ann.

Rev. Pharmacol. Toxicol. 32:573-596; Mulligan, 1993, Science 260:926-932; and Morgan and Anderson, 1993, Ann. Rev. Biochem. 62:191-217; May, 1993, TIBTECH 11(5):155-215. Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel et al. (eds.), 1993, Current Protocols in Molecular Biology, John Wiley & Sons, NY; and Kriegler, 1990, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY.

In a preferred embodiment of the invention, the therapeutic composition comprises a coding sequence that is part of an expression vector. In particular, such a nucleic acid has a promoter operably linked to the coding sequence, said promoter being inducible or constitutive, and, optionally, tissue-specific. In another specific embodiment, a nucleic acid molecule is used in which the coding sequence and any other desired sequences are flanked by regions that promote homologous recombination at a desired site in the genome, thus providing for intrachromosomal expression of the nucleic acid (Koller and Smithies, 1989, Proc. Natl. Acad. Sci. USA 86:8932-8935; Zijlstra et al., 1989, Nature 342:435-438).

Delivery of the nucleic acid into a patient may be either direct, in which case the patient is directly exposed to the nucleic acid or nucleic acid-carrying vector, or indirect, in which case, cells are first transformed with the nucleic acid *in vitro*, then transplanted into the patient. These two approaches are known, respectively, as *in vivo* or *ex vivo* gene therapy.

In a specific embodiment, the nucleic acid is directly administered *in vivo*, where it is expressed to produce the encoded product. This can be accomplished by any methods known in the art, *e.g.*, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, *e.g.*, by infection using a defective or attenuated retroviral or other viral vector (see U.S. Patent No. 4,980,286), by direct injection of naked DNA, by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, DuPont), by coating with lipids or cell-surface receptors or transfecting agents, by encapsulation in liposomes, microparticles, or microcapsules, by administering it in linkage to a peptide which is known to enter the nucleus, or by administering it in linkage to a ligand subject to receptor-mediated endocytosis (see e.g., Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432) which can be used to target cell types specifically expressing the receptors. In another embodiment, a nucleic acid-ligand complex can be formed in which the ligand comprises a fusogenic viral peptide to disrupt endosomes, allowing the nucleic acid to avoid lysosomal degradation. In yet another

embodiment, the nucleic acid can be targeted *in vivo* for cell specific uptake and expression, by targeting a specific receptor (see, e.g., PCT Publications WO 92/06180 dated April 16, 1992; WO 92/22635 dated December 23, 1992; WO92/20316 dated November 26, 1992; WO93/14188 dated July 22, 1993; WO 93/20221 dated October 14, 1993). Alternatively, the nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination (Koller and Smithies, 1989, Proc. Natl. Acad. Sci. USA 86:8932-8935; Zijlstra et al., 1989, Nature 342:435-438).

In a preferred embodiment of the invention, adenoviruses as viral vectors can be used in gene therapy. Adenoviruses have the advantage of being capable of infecting non-dividing cells (Kozarsky and Wilson, 1993, Current Opinion in Genetics and Development 3:499-503). Other instances of the use of adenoviruses in gene therapy can be found in Rosenfeld et al., 1991, Science 252:431-434; Rosenfeld et al., 1992, Cell 68:143-155; and Mastrangeli et al., 1993, J. Clin. Invest. 91:225-234. Furthermore, adenoviral vectors with modified tropism may be used for cell specific targeting (WO98/40508). Adeno-associated virus (AAV) has also been proposed for use in gene therapy (Walsh et al., 1993, Proc. Soc. Exp. Biol. Med. 204:289-300).

In addition, retroviral vectors (see Miller et al., 1993, Meth. Enzymol. 217:581-599) have been modified to delete retroviral sequences that are not necessary for packaging of the viral genome and integration into host cell DNA. The coding sequence to be used in gene therapy is cloned into the vector, which facilitates delivery of the gene into a patient. More detail about retroviral vectors can be found in Boesen et al., 1994, Biotherapy 6:291-302, which describes the use of a retroviral vector to deliver the *mdr1* gene to hematopoietic stem cells in order to make the stem cells more resistant to chemotherapy. Other references illustrating the use of retroviral vectors in gene therapy are: Clowes et al., 1994, J. Clin. Invest. 93:644-651; Kiem et al., 1994, Blood 83:1467-1473; Salmons and Gunzberg, 1993, Human Gene Therapy 4:129-141; and Grossman and Wilson, 1993, Curr. Opin. in Genetics and Devel. 3:110-114.

Another approach to gene therapy involves transferring a gene to cells in tissue culture. Usually, the method of transfer includes the transfer of a selectable marker to the cells. The cells are then placed under selection to isolate those cells that have taken up and are expressing the transferred gene. Those cells are then delivered to a patient.

In this embodiment, the nucleic acid is introduced into a cell prior to administration *in vivo* of the resulting recombinant cell. Such introduction can be carried out by any method known in the art, including but not limited to transfection, electroporation, lipofection, microinjection, infection with a viral or bacteriophage vector containing the nucleic acid sequences, cell fusion, chromosome-mediated gene transfer, microcell-mediated gene transfer, spheroplast fusion, etc. Numerous techniques are known in the art for the introduction of foreign genes into cells (see *e.g.*, Loeffler and Behr, 1993, Meth. Enzymol. 217:599-618; Cohen et al., 1993, Meth. Enzymol. 217:618-644; Cline, 1985, Pharmac. Ther. 29:69-92) and may be used in accordance with the present invention, provided that the necessary developmental and physiological functions of the recipient cells are not disrupted. The technique should provide for the stable transfer of the nucleic acid to the cell, so that the nucleic acid is expressible by the cell and preferably heritable and expressible by its cell progeny. In a preferred embodiment, the cell used for gene therapy is autologous to the patient.

In a specific embodiment, the nucleic acid to be introduced for purposes of gene therapy comprises an inducible promoter operably linked to the coding sequence, such that expression of the nucleic acid is controllable by controlling the presence or absence of the appropriate inducer of transcription.

Oligonucleotides such as anti-sense RNA and DNA molecules, and ribozymes that function to inhibit the translation of a targeted mRNA, especially its C-terminus are also within the scope of the invention. Anti-sense RNA and DNA molecules act to directly block the translation of mRNA by binding to targeted mRNA and preventing protein translation. In regard to antisense DNA, oligodeoxyribonucleotides derived from the translation initiation site, *e.g.*, between -10 and +10 regions of a nucleotide sequence, are preferred.

The antisense oligonucleotide may comprise at least one modified base moiety which is selected from the group including, but not limited to, 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-

D-mannosylqueosine, 5' -methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Within the scope of the invention are engineered hammerhead motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of target RNA sequences.

Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites ,which include the following sequences, GUA, GUU and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for predicted structural features such as secondary structure that may render the oligonucleotide sequence unsuitable. The suitability of candidate targets may also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using ribonuclease protection assays.

The anti-sense RNA and DNA molecules and ribozymes of the invention may be prepared by any method known in the art for the synthesis of nucleic acid molecules. These include techniques for chemically synthesizing oligodeoxyribonucleotides well known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding the RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which contain suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

Various modifications to the DNA molecules may be introduced as a means of increasing intracellular stability and half-life. Possible modifications include, but are not limited to, the addition of flanking sequences of ribo- or deoxy- nucleotides to the 5' and/or 3' ends of the

molecule or the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within the oligodeoxyribonucleotide backbone.

C. Other Pharmaceutical Compositions

The compounds of the invention, may be administered to a subject *per se* or in the form of a sterile composition or a pharmaceutical composition. Pharmaceutical compositions comprising the compounds of the invention may be manufactured by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes. Pharmaceutical compositions may be formulated in conventional manner using one or more physiologically acceptable carriers, diluents, excipients or auxiliaries that facilitate processing of the active peptides or peptide analogues into preparations which can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For topical administration the compounds of the invention can be formulated as solutions, gels, ointments, creams, suspensions, etc. as are well-known in the art.

Systemic formulations include those designed for administration by injection, *e.g.* subcutaneous, intravenous, intramuscular, intrathecal or intraperitoneal injection, as well as those designed for transdermal, transmucosal, oral or pulmonary administration.

For injection, the compounds of the invention can be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. The solution can contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Alternatively, the compounds can be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art. This route of administration may be used to deliver the compounds to the nasal cavity.

For oral administration, the compounds can be readily formulated by combining the active peptides or peptide analogues with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a

patient to be treated. For oral solid formulations such as, for example, powders, capsules and tablets, suitable excipients include fillers such as sugars, such as lactose, sucrose, mannitol and sorbitol; cellulose preparations such as maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP); granulating agents; and binding agents. If desired, disintegrating agents may be added, such as the cross-linked polyvinylpyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

If desired, solid dosage forms may be sugar-coated or enteric-coated using standard techniques.

For oral liquid preparations such as, for example, suspensions, elixirs and solutions, suitable carriers, excipients or diluents include water, glycols, oils, alcohols, etc. Additionally, flavoring agents, preservatives, coloring agents and the like may be added.

For buccal administration, the compounds may take the form of tablets, lozenges, etc. formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of e.g. gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds may also be formulated in rectal or vaginal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

Alternatively, other pharmaceutical delivery systems may be employed. Liposomes and emulsions are well known examples of delivery vehicles that may be used to deliver peptides and peptide analogues of the invention. Certain organic solvents such as dimethylsulfoxide also may be employed, although usually at the cost of greater toxicity. Additionally, the compounds may be delivered using a sustained-release system, such as semipermeable matrices of solid polymers containing the therapeutic agent. Various of sustained-release materials have been established and are well known by those skilled in the art. Sustained-release capsules may, depending on their chemical nature, release the compounds for a few weeks up to over 100 days. Depending on the chemical nature and the biological stability of the therapeutic reagent, additional strategies for protein stabilization may be employed.

As the compounds of the invention may contain charged side chains or termini, they may be included in any of the above-described formulations as the free acids or bases or as pharmaceutically acceptable salts. Pharmaceutically acceptable salts are those salts which substantially retain the biologic activity of the free bases and which are prepared by reaction with inorganic acids. Pharmaceutical salts tend to be more soluble in aqueous and other protic solvents than are the corresponding free base forms.

D. Effective Dosages

The compounds of the invention will generally be used in an amount effective to achieve the intended purpose (e.g., treatment of a neuronal injury). The compounds of the invention or pharmaceutical compositions thereof, are administered or applied in a therapeutically effective amount. By therapeutically effective amount is meant an amount effective ameliorate or prevent the symptoms, or prolong the survival of, the patient being treated. Determination of a therapeutically effective amount is well within the capabilities of those skilled in the art, especially in light of the detailed disclosure provided herein. An "inhibitory amount" or "inhibitory concentration" of a PL-PDZ binding inhibitor is an amount that reduces binding by at least about 40%, preferably at least about 50%, often at least about 70%, and even as much as at least about 90%. Binding can be measured *in vitro* (e.g., in an A assay or G assay) or in situ.

For systemic administration, a therapeutically effective dose can be estimated initially from *in vitro* assays. For example, a dose can be formulated in animal models to

achieve a circulating concentration range that includes the IC_{50} as determined in cell culture. Such information can be used to more accurately determine useful doses in humans.

Initial dosages can also be estimated from *in vivo* data, *e.g.*, animal models, using techniques that are well known in the art. One having ordinary skill in the art could readily optimize administration to humans based on animal data.

Dosage amount and interval may be adjusted individually to provide plasma levels of the compounds that are sufficient to maintain therapeutic effect. Usual patient dosages for administration by injection range from about 0.1 to 5 mg/kg/day, preferably from about 0.5 to 1 mg/kg/day. Therapeutically effective serum levels may be achieved by administering multiple doses each day. For usual peptide therapeutic treatment of stroke, acute administration of 0.03 nmol/g to 30 nmol/g within 6 hours of stroke or brain ischemia is typical. In other instances, 0.1 nmol/g to 20 nmol/g within 6 hours are administered. And in still other instances 1nmol/g to 10 nmol/g is administered with in 6 hours.

In cases of local administration or selective uptake, the effective local concentration of the compounds may not be related to plasma concentration. One having skill in the art will be able to optimize therapeutically effective local dosages without undue experimentation.

The amount of compound administered will, of course, be dependent on the subject being treated, on the subject's weight, the severity of the affliction, the manner of administration and the judgment of the prescribing physician.

The therapy may be repeated intermittently while symptoms detectable or even when they are not detectable. The therapy may be provided alone or in combination with other drugs.

E. Toxicity

Preferably, a therapeutically effective dose of the compounds described herein will provide therapeutic benefit without causing substantial toxicity.

Toxicity of the compounds described herein can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, by determining the LD_{50} (the dose lethal to 50% of the population) or the LD_{100} (the dose lethal to 100% of the population). The dose ratio between toxic and therapeutic effect is the therapeutic index.

Compounds which exhibit high therapeutic indices are preferred. The data obtained from these cell culture assays and animal studies can be used in formulating a dosage range that is not toxic for use in human. The dosage of the compounds described herein lies preferably within a range of circulating concentrations that include the effective dose with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (*See, e.g.,* Fingl et al., 1975, In: *The Pharmacological Basis of Therapeutics*, Ch.1, p.1).

Examples

EXAMPLE 1

IDENTIFICATION OF TRPM7 INTERACTIONS WITH PDZ DOMAINS

This example describes assays to identify binding between TRPM7 and PDZ domains. GST-PDZ fusions are produced that each contain an entire PDZ domain (or multiple PDZ domains), the collection encompassing approximately 90% of all PDZ domains identified in the human genome. (See Table 5 and Section V (PDZ Proteins and PL Protein Interactions Disclosure). Biotinylated peptide corresponding to 11 residues of Tat coupled to the C-terminal 9 amino acids of TRPM7 (YGRKKRRQRRRSTNSVRLML; SEQ ID NO:258) is synthesized and purified by HPLC.

Binding between these entities is detected through the G or the G3 Assays, colorimetric assays using avidin-HRP to bind the biotin and a peroxidase substrate.

Another type of assay is a fluorescence-based binding assay using the rhodamine derivative TAMRA (Molecular Probes) conjugated to the C-terminal 9 amino acids of TRPM7 (TAMRA-RRSTNSVRLML; SEQ ID NO:259).

A. G Assay for Identification of Interactions Between Peptides and Fusion Proteins

Reagents and Materials:

- Nunc Maxisorp 96 well Immuno-plate
- PBS pH 7.4 (Gibco BRL Cat #16777-148) or (AVC phosphate buffered saline, 8g NaCl, 0.29 g KCl, 1.44 g Na_2HPO_4, 0.24g KH_2PO_4, add H_2O to 1 liter and pH 7.4; 0.2 μm filter)
- 2% BSA/PBS (10g of bovine serum albumin, fraction V (ICN Biomedicals Cat. # IC15142983) into 500 ml PBS
- Goat anti-GST mAb stock @ 5 mg/ml, store at 4°C, (Amersham Pharmacia Cat.#27-4577-01), dilute 1:1000 in PBS, final concentration 5 μg/ml
- HRP-Streptavidin, 2.5mg/2ml stock stored at 4°C (Zymed Cat. #43-4323), dilute 1:2000 into 2% BSA, final concentration at 0.5 μg/ml
- Wash Buffer, PBS
- TMB ready to use (Dako Cat #S1600)
- 1M H_2SO_4
- 12w multichannel pipettor,
- 50 ml reagent reservoirs,
- 15 ml polypropylene conical tubes

Protocol

1) Coat plate with 100 μl of 5 μg/ml goat anti GST, overnight at 4°C
2) Dump coating antibodies out and tap dry
3) Blocking - Add 200 μl per well 2% BSA, 2 hrs at room temperature
4) Prepare proteins at 5 μg/ml in 2% BSA (2 ml per row or per two columns)
5) 3 washes with cold PBS (must be cold through entire experiment)
   (at last wash leave PBS in wells until immediately adding next step)
6) Add proteins at 50 μl per well on ice (1 to 2 hrs at 4°C)
7) Prepare Peptides at desired concentration in 2% BSA (2 ml/row or /columns)
8) 3 X wash with cold PBS
9) Add peptides at 50 μl per well on ice (time on / time off)
   a. keep on ice after last peptide has been added for 10 minutes exactly
   b. place at room temp for 20 minutes exactly
10) Prepare 12 ml/plate of HRP-Streptavidin (1:2000 dilution in 2% BSA)

11) 3 X wash with cold PBS, (must be cold, critical)
12) Add HRP-Streptavidin at 100 µl per well on ice, 20 minutes at 4°C
13) Turn on plate reader
14) 5 X washes with PBS at room temperature, avoid bubbles
15) Add TMB substrate at 100 µl per well
    a. *incubate in dark at room temperature*
    b. check plate periodically (5, 10, & 20 minutes)
    c. take early readings, if necessary, at 650 nm (blue)
    d. at 30 minutes, stop reaction with 100 µl of 1M H_2SO_4
    e. take last reading at 450 nm (yellow

B. G3 ASSAY FOR IDENTIFICATION OF INTERACTIONS BETWEEN PEPTIDES AND FUSION PROTEINS

REAGENTS AND SUPPLIES

- Nunc MaxiSorp 96 well Immuno-plate, Nunc
- PBS pH 7.4 (phosphate buffered saline, 8g NaCl, 0.29g KCl, 1.44g Na_2HPO_4, 0.24g KH_2PO_4, add H_2O to 1 liter and pH 7.4; 0.2 µm filter)
- Assay Buffer: 2% BSA in PBS (20g of bovine serum albumin per liter PBS, fraction V, ICN Biomedicals, cat#IC15142983
- Goat anti-GST polyclonal Ab, stock 5 mg/ml, stored at 4°C, Amersham Pharmacia cat#27-4577-01
    - Dilute 1:1000 in PBS, final concentration 5 µg/ml.
- HRP-Streptavidin, 2.5mg/2ml stock stored at 4°C, Zymed cat#43-4323, dilute 1:2000 into Assay buffer, final concentration of 0.5 µg/ml
- Wash Buffer, PBS
- Biotinylated peptides (HPLC purified, stock solution stored in -20°C freezer)
- GST-PRISM proteins (stock stored at -80°C, after 1st thaw store in -10°C freezer)
- TMB (3,3',5,5', teramethylbensidine), tablets, Sigma cat.#T5525
    - *Per plate, dissolve 1 tablet in 1ml DMSO, add 9ml Citrate/Phosphate buffer pH 5.4 and 2µL H_2O_2*
- 0.18M H_2SO_4, Sigma cat.#S1526
- 12-w multichannel pipettor & tips
- 50 ml reagent reservoirs, Costar#4870
- 50, 15 ml polypropylene conical tubes
- Costar Transtar 96 Costar #7605
- Transtar 96 Cartridge Costar #7610

- Molecular Devices microplate reader (450 & 650 nm filters) with SoftMax Pro software

*When using reagents stored at or 4°C or -20°C, remove & keep on ice

PROTOCOL

1. Coat plate with 100 µl of 5 µg/ml anti-GST, overnight at 4°C
2. Dump contents of plate & out tap dry on paper towels
3. Block with 200µl Assay Buffer for 2 hrs at room temperature
4. Prepare proteins at 5 µg/ml in Assay Buffer
5. Wash 3X with cold PBS (4°C), avoiding letting the plates dry out
6. Add proteins at 50 µl per well, incubate 1 to 2 hrs at 4°C
7. Prepare peptides to desired concentration in Assay Buffer
8. Wash 3X with cold PBS (4°C), avoiding letting the plates dry out
9. Add HRP-Streptavidin/peptide complex [prepared by incubating for 20 minutes at room temperature HRP-Streptavidin (1:2000) with the desired biotin-peptide concentration for 20 minutes at room temperature] at 50 µl per well on ice
10. Place at room temperature for exactly 30 minutes
11. Promptly wash 5X with PBS at room temperature
12. Add 100 µl/well TMB substrate (write time on plate)
13. Incubate in dark at room temperature for a maximum of 30 minutes
14. Read plate at 25 minutes (650 nm), *optional*
15. Stop reaction with 100 µl of 0.18 M H_2SO_4, 30 minutes after adding TMB

Take last reading at 450 nm soon after stopping reaction

C. Results of Binding Experiments

The G3 assay was performed using 0.1 µM peptide representing 11 amino acids of Tat fused to the final 9 amino acids of TRPM7 (YGRKKRRQRRRSTNSVRLML; SEQ ID NO:258). Table 8 shows the PDZ domains which bound the peptide with an OD at least 2-fold over GST-only OD in the G3 assay as described above and/or had an ED_{50} of less than 0.17 µM in the titration experiments described in Example 2 below.

EXAMPLE 2

TITRATION EXPERIMENTS

The G3 assay was performed on a subset of the PDZ domains as in Example II except that the concentration of peptide was 0.002 µM, 002µM, 0.1 µM, 0.2 µM, 1 µM, and 2 µM. Data was fitted to the equation:

$y=100.0/(1+10^{((\log(m1)-\log(m0))*m2)})$, where m1=$EC_{50}$, m2=Hill coefficient, and m0=peptide concentration

to calculate the EC_{50} for the binding of the peptides to the PDZ domains.

Results:

EC_{50} data are presented in Table 9. Graphs of titrations for the biotinylated peptide representing 11 amino acids of Tat fused to the final 9 amino acids of TRPM7 (YGRKKRRQRRRSTNSVRLML; SEQ ID NO:258) peptide with RIM-2 d1, INADL d3, ZO-1 d2, and Par3 d3 are shown in Figures 9A, 9B, 9C, and 9D.

EXAMPLE 3

PEPTIDE BINDING

The G3 assay was performed to determine the binding of PDZ domains ZO-1 d2 and INADL d3 with the peptides listed in Table 10. EC_{50} results are shown in Table 10. Figures 10A and 10B show graphs of the titrations for the Peptide #1829 with ZO-1 d2 and for the Peptide #1839 with INADL d3.

The peptides shown in Table 11 have also been demonstrated to bind ZO-1 d2, INADL d3, PAR3 d3, syntrophin 1 alpha d1, and RIM2 d1 in G assays.

EXAMPLE 4

TREATMENT OF ISCHEMIC BRAIN DAMAGE BY MODULATING TRPM7 EXPRESSION

Generation of a DNA vector-based siRNA system.

In brief, a small DNA insert (49 bp) encoding a short hairpin RNA targeting TRPM7 (the siRNA against TRPM7 corresponded to coding regions 5152-5172 ($siRNA_{TRPM7-1}$)) was cloned into a commercially available pAdTrack vector (see Figure 11). The sequence was placed under the control of the H1 promoter and GFP was placed under a CMV promoter. The TRPM7 siRNA-pAdTrack insert-containing vector as well as adeno recombination sequences were co-transfected with pAdEasy (containing viral sequences) into HEK cells and the cells were

selected with kanamycin. Recombined virus containing the TRPM7 siRNA and GFP sequences was produced from these cells. The hairpin RNA was rapidly processed by the cellular machinery into 45 nt double stranded RNA (siRNA). In this way, the siRNA was delivered using a viral vector. Two additional effective RNAi sequences for TRPM7 include siRNAs against TRPM7 corresponding to coding regions, 5023-5043 and 1318-1338 ($siRNA_{TRPM7-2}$ and $siRNA_{TRPM7-3}$, respectively) relative to the first nucleotide of the start codon of murine TRPM7 (GeneBank accession # AY032951).

Figure 12 shows the effects of infecting primary cultured neurons on TRPM7 mRNA by RT-PCR. Cultured cortical neurons plated in 12 well plates (106 cells/well) were infected at the time of plating with the corresponding adenoviral constructs and the RNA was harvested 5 days later. RT-PCR was performed on equivalent amounts of RNA using primers for TRPM7 or β-actin.

Figure 13 shows the effect of oxygen glucose deprivation (OGD) for the indicated duration on neuronal cell death in the presence (MCN(+)) or absence (MCN(-)) of the combination of MK-801 (10 μM), CNQX (10 μM) and nimodipine (2 μM), denoted as MCN, antagonists of NMDA and AMPA/kainate glutamate receptors and L-type Ca^{2+} channels, respectively. The fraction of dead cells was determining by dividing the number of neurons expressing GFP which became stained with propidium iodide 20h after OGD by the number of neurons expressing GFP at the beginning of the experiment. Data for each culture was obtained from counting cells in 3 high power microscope fields per experiment.

The siRNA sequences for TRPM7 may also be placed under the control of the human U6 promoter in the shuttle vector pTrack CMV-EGFP, whereby the siRNA sequences and the EGFP sequence will be driven off separate promoters. A control adenovirus expressing EGFP alone, using the same shuttle vector as above, will alsobe produced.

Regional infection of rat cortex and hippocampus with RNAi adenoviruses by microinjection.

3 μl of either TRPM7 vector or control vector are infused to the cortex (from bregma: AP = 2 mm, ML = 2.8 mm, DV = 1.7 mm) or in the CA1 sector of the hippocampus. We infuse in the order of 10^4 particles. Animals are sacrificed 2, 5 and 10 days later and brain

sections prepared and analyzed for GFP fluorescence in frozen brain sections and for GFP staining with anti-GFP antibodies in fixed sections. The technique can be titrated (amount of infectious particles, duration of treatment, number of treatments) to optimize TRPM7 suppression. All animals undergo bilateral injections, using the active vector on one side, and the control on the other, to control for effects of the adenovirus.

Effect of in-vivo TRPM7 suppression on cerebral ischemic damage.

Experiments are performed using a permanent distal middle cerebral artery occlusion (MCAO) model as described by Brint et al.(1988). This results primarily in the death of cortical neurons. In brief, Wistar rats (250-300 g) are anesthetized with 1-2% halothane. Snares are placed around the CCAs and a 2 mm burr hole made at the junction of the zygomatic arch and squamous bone. The distal MCA is exposed and divided above the rhinal fissure. The CCA snares is tightened to occlude the CCAs, and released after 4h.

Experiments are performed in animals in which TRPM7 was suppressed as in **5.2** bilaterally in the cortex. Animals are sacrificed 48 hr later and brain sections prepared and analyzed. Neuronal cell loss will be assessed in transfected areas as gauged by GFP expression, which is co-expressed with siRNA in the same vector and for infarct volumes. Experimental groups will include animals receiving injections of saline (10), Scrambled (inactive) RNAi vector (10), and TRPM7 RNAi vector (10). The hemisphere contralateral to the MCAO will serve as a control in each animal. If TRPM7 suppression is protective, then there will be improved survival of neurons in transfected (GFP positive) areas.

EXAMPLE 5:

TREATMENT OF ISCHEMIC BRAIN DAMAGE BY MODULATING TRPM7-PDZ PROTEIN INTERACTIONS

Small peptides or fusion proteins containing C- and N-terminus TRPM7 subunit sequences, or encompassing intrinsic TRPM channel enzymatic domains will be synthesized or grown in bacteria. Some will be grown as Flag- or HA-tagged proteins fused to a peptide corresponding to the cell-membrane transduction domain of the HIV-1-Tat protein

(YGRKKRRQRRR; Tat; SEQ ID NO:257). Tat-peptides and proteins permeate into cells in a rapid, dose-dependent manner independent of receptors and transporters.

Figure 14 shows the effect of treating the cultures with Tat-9cTRPM7. The sequence of Tat-9cTRPM7 is: [YGRKKRRQRRR-STNSVRLML; SEQ ID NO:258], whereby the first 11 residues correspond to the cell-membrane transduction domain of the human immunodeficiency virus type 1 (HIV-1) Tat protein and the last 9 residues correspond to the last 9 amino acids of the C-terminus of human TRPM7 (accession Q96QT4). (A) Neuronal survival at 20h in the indicated concentrations of Tat-9cTRPM7 in the absence of excitotoxic challenge. (B) Neuronal survival 20h after challenging the cultures for 1h with the indicated concentration of NMDA. Tat-9cTRPM7 was applied immediately after the NMDA challenge. Therefore, neurons treated with 9C-TRPM7 exhibited enhanced survival when challenged with NMDA.

We predict similar results with a Tat-conjugated peptide encoding the last 9 residues of the mouse TRPM7 C-terminus (YGRKKRRQRRR-ATNSVRLML; SEQ ID NO:380; accession Q923J1).

To examine whether treatment with these Tat-conjugated TRPM7 C-terminal peptides would reduce stroke damage, experiments are carried out in adult male Sprague-Dawley rats subjected to transient middle cerebral artery occlusion (MCAO) for 90 minutes by the intraluminal suture method. This produces an extensive infarction encompassing the cerebral cortex and basal ganglia. Animals are treated with either saline, the Tat-TRPM7 C-terminus, or with Tat-conjugated to a scrambled C-terminal peptide by a single intravenous bolus injection 1 hour after the onset of MCAO (3 nMoles/g). Physiological parameters (body temperature, blood pressure, blood gases) are monitored and maintained throughout the experiment. All experimental manipulations and analyses of data are performed by individuals blinded to the treatment groups. The extent of cerebral infarction is measured 24h after MCAO onset (Fig. 3C inset). The postural reflex test, and the forelimb placing test is used to grade neurological function on a scale of 0 to 12 (normal = 0; worst = 12) during MCAO (at 50 minutes) and 24h thereafter.

***

The present invention is not to be limited in scope by the exemplified embodiments which are intended as illustrations of single aspects of the invention and any sequences which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

All publications, patents and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes to the same extent as if each individual publication, patent or patent application were specifically and individually indicated to be so incorporated by reference.